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NUCLEOTIDE AND PROTEIN SEQUENCES OF VERTEBRATE DELTA GENES AND METHODS BASED THEREON

This application claims priority to United States
Provisional Application Serial No. 60/000,589 filed June 28,
1995, which is incorporated by reference herein in its
entirety.

1. INTRODUCTION

The present invention relates to vertebrate Delta genes and their encoded protein products, as well as derivatives and analogs thereof. Production of vertebrate Delta proteins, derivatives, and antibodies is also provided. The invention further relates to therapeutic compositions and methods of diagnosis and therapy.

2. BACKGROUND OF THE INVENTION

Genetic analyses in Drosophila have been extremely useful in dissecting the complexity of developmental pathways and identifying interacting loci. However, understanding the precise nature of the processes that underlie genetic interactions requires a knowledge of the protein products of the genes in question.

The vertebrate central nervous system is an

25 intimate mixture of different cell types, almost all
generated from the same source - the neurogenic epithelium
that forms the neural plate and subsequently the neural tube.
What are the mechanisms that control neurogenesis in this
sheet of cells, directing some to become neurons while others

30 remain non-neuronal? The answer is virtually unknown for
vertebrates, but many of the cellular interactions and genes
controlling cell fate decisions during neurogenesis have been
well characterized in Drosophila (Campos-Ortega, 1993, J.
Neurobiol. 24:1305-1327). Although the gross anatomical

35 context of neurogenesis appears very different in insects and
vertebrates, the possibility remains that, at a cellular
level, similar events are occurring via conserved molecular



mechanisms. Embryological, genetic and molecular evidence indicates that the early steps of ectodermal differentiation in *Drosophila* depend on cell interactions (Doe and Goodman, 1985, Dev. Biol. 111:206-219; Technau and Campos-Ortega,

- 5 1986, Dev. Biol. 195:445-454; Vässin et al., 1985, J.
 Neurogenet. 2:291-308; de la Concha et al., 1988, Genetics
 118:499-508; Xu et al., 1990, Genes Dev. 4:464-475;
 Artavanis-Tsakonas, 1988, Trends Genet. 4:95-100).
 Mutational analyses reveal a small group of zygotically-
- 10 acting genes, the so called neurogenic loci, which affect the choice of ectodermal cells between epidermal and neural pathways (Poulson, 1937, Proc. Natl. Acad. Sci. 23:133-137; Lehmann et al., 1983, Wilhelm Roux's Arch. Dev. Biol. 192:62-74; Jürgens et al., 1984, Wilhelm Roux's Arch. Dev. Biol.
- 15 193:283-295; Wieschaus et al., 1984, Wilhelm Roux's Arch.

 Dev. Biol. 193:296-307; Nüsslein-Volhard et al., 1984,

 Wilhelm Roux's Arch. Dev. Biol. 193:267-282). Null mutations

 in any one of the zygotic neurogenic loci -- Notch (N), Delta

 (D1), mastermind (mam), Enhancer of Split (E(spl), neuralized)
- 20 (neu), and big brain (bib) -- result in hypertrophy of the nervous system at the expense of ventral and lateral epidermal structures. This effect is due to the misrouting of epidermal precursor cells into a neuronal pathway, and implies that neurogenic gene function is necessary to divert
- 25 cells within the neurogenic region from a neuronal fate to an epithelial fate.

Neural precursors arise in the *Drosophila* embryo from a neurogenic epithelium during successive waves of neurogenesis (Campos-Ortega & Hartenstein, 1985, The

- 30 embryonic development of Drosophila melanogaster (Springer-Verlag, Berlin; New York); Doe, 1992, Development 116:855-863). The pattern of production of these cells is largely determined by the activity of the proneural and neurogenic genes. Proneural genes predispose clusters of
- 35 cells to a neural fate (reviewed in Skeath & Carroll, 1994, Faseb J. 8:714-21), but only a subset of cells in a cluster become neural precursors. This restriction is due to the



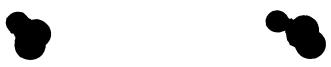
action of the neurogenic genes, which mediate lateral inhibition - a type of inhibitory cell signaling by which a cell committed to a neural fate forces its neighbors either to remain uncommitted or to enter a non-neural pathway

- 5 (Artavanis-Tsakonas & Simpson, 1991, Trends Genet. 7:403-408; Doe & Goodman, 1985, Dev. Biol. 111:206-219). Mutations leading to a failure of lateral inhibition cause an overproduction of neurons the "neurogenic" phenotype (Lehmann et al., 1981, Roux's Arch. Dev. Biol. 190:226-229;
- 10 Lehmann et al., Roux's Arch. Dev. Biol. 192:62-74). In Drosophila, the inhibitory signal is delivered by a transmembrane protein encoded by the Delta neurogenic gene, which is displayed by the nascent neural cells (Heitzler & Simpson, 1991, Cell 64:1083-1092). Neighboring cells express
- 15 a transmembrane receptor protein, encoded by the neurogenic gene Notch (Fortini & Artavanis-Tsakonas, 1993, Cell 75:1245-1247). Delta has been identified as a genetic unit capable of interacting with the Notch locus (Xu et al., 1990, Genes Dev. 4:464-475).
- Mutational analyses also reveal that the action of the neurogenic genes is pleiotropic and is not limited solely to embryogenesis. For example, ommatidial, bristle and wing formation, which are known also to depend upon cell interactions, are affected by neurogenic mutations (Morgan et
- 25 al., 1925, Bibliogr. Genet. 2:1-226; Welshons, 1956, Dros.
 Inf. Serv. 30:157-158; Preiss et al., 1988, EMBO J. 7:3917 3927; Shellenbarger and Mohler, 1978, Dev. Biol. 62:432-446;
 Technau and Campos-Ortega, 1986, Wilhelm Roux's Dev. Biol.
 195:445-454; Tomlison and Ready, 1987, Dev. Biol. 120:366-
- 30 376; Cagan and Ready, 1989, Genes Dev. 3:1099-1112).

 Neurogenic genes are also required for normal development of the muscles, gut, excretory and reproductive systems of the fly (Muskavitch, 1994, Dev. Biol. 166:415-430).

Both Notch and Delta are transmembrane proteins

35 that span the membrane a single time (Wharton et al., 1985, Cell 43:567-581; Kidd and Young, 1986, Mol. Cell. Biol. 6:3094-3108; Vässin, et al., 1987, EMBO J. 6:3431-3440;



Kopczynski, et al., 1988, Genes Dev. 2:1723-1735) and include multiple tandem EGF-like repeats in their extracellular domains (Muskavitch, 1994, Dev. Biol. 166:415-430). The Notch gene encodes a ~300 kd protein (we use "Notch" to

- 5 denote this protein) with a large N-terminal extracellular domain that includes 36 epidermal growth factor (EGF)-like tandem repeats followed by three other cysteine-rich repeats, designated Notch/lin-12 repeats (Wharton, et al., 1985, Cell 43:567-581; Kidd and Young, 1986, Mol. Cell. Biol. 6:3094-
- 10 3108; Yochem, et al., 1988, Nature 335:547-550). Molecular studies have lead to the suggestion that Notch and Delta constitute biochemically interacting elements of a cell communication mechanism involved in early developmental decisions (Fehon et al., 1990, Cell 61:523-534). Homologs
- gene lin-12 and the Delta-related gene lag-2 are also responsible for lateral inhibition (Sternberg, 1993, Current Biol. 3:763-765; Henderson et al., 1994, Development 120:2913-2924; Greenwald, 1994, Curr. Opin. Genet. Dev.
- 20 4:556-562). In vertebrates, several Notch homologs have also been identified (Kopan & Weintraub, 1993, J. Cell Biol. 121:631-641; Lardelli et al., 1994, Mech. Dev. 46:123-136; Lardelli & Lendahl, 1993, Exp. Cell Res. 204:364-372; Weinmaster et al., 1991, Development 113:199-205; Weinmaster
- 25 et al., 1992, Development 116:931-941; Coffman et al., 1990, Science 249:1438-1441; Bierkamp & Campos-Ortega, 1993, Mech. Dev. 43:87-100), and they are expressed in many tissues and at many stages of development. Loss of Notch-1 leads to somite defects and embryonic death in mice (Swiatek et al.,
- 30 1994, Genes Dev. 8:707-719; Conlon et al., Rossant, J. Development (J. Dev. 121:1533-1545), while constitutively active mutant forms of Notch-1 appear to inhibit cell differentiation in Xenopus and in cultured mammalian cells (Coffman et al., 1993, Cell 73:659-671; Kopan et al., 1994,
- 35 Development 120:2385-2396; Nye et al., 1994, Development 120:2421-2430).

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The EGF-like motif has been found in a variety of proteins, including those involved in the blood clotting cascade (Furie and Furie, 1988, Cell 53: 505-518). In particular, this motif has been found in extracellular

- 5 proteins such as the blood clotting factors IX and X (Rees et al., 1988, EMBO J. 7:2053-2061; Furie and Furie, 1988, Cell 53: 505-518), in other *Drosophila* genes (Knust et al., 1987 EMBO J. 761-766; Rothberg et al., 1988, Cell 55:1047-1059), and in some cell-surface receptor proteins, such as
- thrombomodulin (Suzuki et al., 1987, EMBO J. 6:1891-1897) and LDL receptor (Sudhof et al., 1985, Science 228:815-822). A protein binding site has been mapped to the EGF repeat domain in thrombomodulin and urokinase (Kurosawa et al., 1988, J. Biol. Chem 263:5993-5996; Appella et al., 1987, J. Biol.
 15 Chem. 262:4437-4440).

Citation of references hereinabove shall not be construed as an admission that such references are prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention relates to nucleotide sequences of vertebrate *Delta* genes (chick and mouse *Delta*, and related genes of other species), and amino acid sequences of their encoded proteins, as well as derivatives (e.g.,

25 fragments) and analogs thereof. Nucleic acids hybridizable to or complementary to the foregoing nucleotide sequences are also provided. In a specific embodiment, the Delta protein is a mammalian protein, preferably a human protein.

The invention relates to vertebrate Delta

- 30 derivatives and analogs of the invention which are functionally active, i.e., they are capable of displaying one or more known functional activities associated with a full-length (wild-type) Delta protein. Such functional activities include but are not limited to antigenicity [ability to bind
- 35 (or compete with Delta for binding) to an anti-Delta antibody], immunogenicity (ability to generate antibody which binds to Delta), ability to bind (or compete with Delta for





binding) to Notch or other toporythmic proteins or fragments thereof ("adhesiveness"), ability to bind (or compete with Delta for binding) to a receptor for Delta. "Toporythmic proteins" as used herein, refers to the protein products of Notch, Delta, Serrate, Enhancer of split, and Deltex, as well as other members of this interacting set of genes which may be identified, e.g., by virtue of the ability of their gene sequences to hybridize, or their homology to Delta, Serrate, or Notch, or the ability of their genes to display phenotypic interactions or the ability of their protein products to interact biochemically.

The invention further relates to fragments (and derivatives and analogs thereof) of a vertebrate Delta that comprise one or more domains of the Delta protein, including but not limited to the intracellular domain, extracellular domain, transmembrane domain, DSL domain, domain aminoterminal to the DSL domain, or one or more EGF-like (homologous) repeats of a Delta protein, or any combination of the foregoing.

Antibodies to a vertebrate Delta, its derivatives and analogs, are additionally provided.

Methods of production of the vertebrate Delta proteins, derivatives and analogs, e.g., by recombinant means, are also provided.

- 25 The present invention also relates to therapeutic and diagnostic methods and compositions based on Delta proteins and nucleic acids. The invention provides for treatment of disorders of cell fate or differentiation by administration of a therapeutic compound of the invention.
- 30 Such therapeutic compounds (termed herein "Therapeutics") include: Delta proteins and analogs and derivatives (including fragments) thereof; antibodies thereto; nucleic acids encoding the Delta proteins, analogs, or derivatives; and Delta antisense nucleic acids. In a preferred
- 35 embodiment, a Therapeutic of the invention is administered to treat a cancerous condition, or to prevent progression from a pre-neoplastic or non-malignant state into a neoplastic or a



malignant state. In other specific embodiments, a
Therapeutic of the invention is administered to treat a
nervous system disorder or to promote tissue regeneration and
repair.

- In one embodiment, Therapeutics which antagonize, or inhibit, Notch and/or Delta function (hereinafter "Antagonist Therapeutics") are administered for therapeutic effect. In another embodiment, Therapeutics which promote Notch and/or Delta function (hereinafter "Agonist
- 10 Therapeutics") are administered for therapeutic effect.

 Disorders of cell fate, in particular
 hyperproliferative (e.g., cancer) or hypoproliferative
 disorders, involving aberrant or undesirable levels of
 expression or activity or localization of Notch and/or Delta

15 protein can be diagnosed by detecting such levels, as described more fully infra.

In a preferred aspect, a Therapeutic of the invention is a protein consisting of at least a fragment (termed herein "adhesive fragment") of Delta which mediates 20 binding to a Notch protein or a fragment thereof.

3.1. DEFINITIONS

As used herein, underscoring or italicizing the name of a gene shall indicate the gene, in contrast to its

25 encoded protein product which is indicated by the name of the gene in the absence of any underscoring. For example,

"Delta" shall mean the Delta gene, whereas "Delta" shall indicate the protein product of the Delta gene.

4. DESCRIPTION OF THE FIGURES

Figure 1A-1B. 1A. The DNA sequence of chick Delta (C-Delta-1) (SEQ ID NO:1). 1B. The DNA sequence of an alternatively spliced chick Delta (C-Delta-1) (SEQ ID NO:3).

Figure 2. The predicted amino acid sequence of chick 35 Delta (C-Delta-1) (SEQ ID NO:2).

Figure 3. Predicted amino acid sequence of C-Delta-1 (SEQ ID NO:2), aligned with that of X-Delta-1 (Xenopus Delta;





SEQ ID NO:5) and Drosophila Delta (SEQ ID NO:6) and, indicating the conserved domain structures: EGF repeats, DSL domain, and transmembrane domain (TM). Conserved amino acids are boxed, and ● denote aligned and non-aligned N-terminal cysteine residues, respectively. Although the intracellular domains of C-Delta-1 and X-Delta-1 closely resemble each other, they show no significant homology to the corresponding part of Drosophila Delta.

Figure 4. Alignment of DSL domains from C-Delta-1 (SEQ 10 NO:2), Drosophila Delta (SEQ ID NO:6) (Vässin et al., 1987, EMBO J. 6:3431-3440; Kopczynski et al., 1988, Genes Dev. 2:1723-1735), Drosophila Serrate (SEQ ID NO:7) (Fleming et al., 1990, Genes Dev. 4:2188-2201; Thomas et al., 1991, Development 111:749-761), C-Serrate-1 (SEQ ID NO:8) (Myat, 15 Henrique, Ish-Horowicz and Lewis, in preparation), Apx-1 (SEQ ID NO:9) (Mello et al., 1994, Cell 77:95-106) and Lag-2 (SEQ ID NO:10) (Henderson et al., 1994, Development 120:2913-2924; Tax et al., 1994, Nature 368:150-154), showing the conserved Cysteine spacings, the amino acids that are conserved between presumed ligands for Notch-like proteins in Drosophila and vertebrates, and those that are further conserved in C. elegans ligands (boxes).

Figure 5A-5E. C-Delta-1 and C-Notch-1 expression correlate with onset of neurogenesis in the one-day (E1)

25 neural plate. Anterior is to the left. Wholemount in situ hybridization specimens are shown in Figure 5a-d; 5e is a section. Figure 5a, At stage 7, C-Notch-1 is expressed throughout most of the neural plate and part of the underlying presomitic mesoderm. Figure 5b, C-Delta-1 at

30 stage 7 is already detectable in the neural plate, in the future posterior hindbrain, just anterior to the first somite (white box). The posterior end of this neural domain is roughly level with the anterior margin of a domain of very strong expression in the underlying presomitic mesoderm

35 (psm). Earlier expression in the neural plate may occur and be masked by expression in the underlying mesoderm

(unpublished results). Figure 5c, Higher magnification view

of the area boxed in 5b, showing scattered cells in the neural plate expressing C-Delta-1. Figure 5d, At stage 8, C-Delta-1 expression in the neural plate extends posteriorly as the neural plate develops. The domain of labelled neural plate cells visible in this photograph (bracketed) continues posteriorly over the presomitic mesoderm. Figure 5e, Parasagittal section of a stage 8 embryo showing that C-Delta-1 is expressed in scattered cells of the neural plate (dorsal layer of tissue; bracketed), and broadly in the presomitic mesoderm (ventral layer). The plane of section is slightly oblique, missing the posterior part of the neural plate domain (cf. 5d).

Figure 6A-6C. C-Delta-1-expressing cells do not incorporate BrdU. Of 612 C-Delta-1* cells, 581 were BrdU⁻ (76 15 sections; 6 embryos). Figure 6a, Diagram showing how phase in the cell cycle is related to apico-basal position of the nucleus for cells in the neuroepithelium; S-phase nuclei lie basally (Fujita, 1963, J. Comp. Neurol. 120:37-42; Biffo et al., 1992, Histochem. Cytochem. 40:535-540). Nuclei are

- 20 indicated by shading. Figure 6b, Section through the neural tube of a stage 9 embryo labelled for 2 h with BrdU showing C-Delta-1 expressing cells (dark on blue background) and BrdU-labelled nuclei (pink). Labelled nuclei are predominantly basal, where DNA synthesis occurs, yet basal
- 25 C-Delta-1-expressing cells are unlabelled. Figure 6c, Section through a stage 9 embryo incubated for 4h: many labelled nuclei have exited S-phase and have moved towards the lumen, but C-Delta-1-expressing cells are still basal and not labelled with BrdU.

Figure 7. The DNA sequence of mouse Delta (M-Delta-1) (SEQ ID NO:11).

Figure 8. The predicted amino acid sequence of the mouse Delta (M-Delta-1) (SEQ ID NO:12).

Figure 9. An alignment of the predicted amino acid

35 sequence of mouse M-Delta-1 (SEQ ID NO:12) with the chick C
Delta-1 (SEQ ID NO:2) which shows their extensive amino acid

sequence identity. Identical amino acids are boxed. The

consensus sequence between the two genes is at the bottom (SEQ ID NO:13).

Figure 10. The DNA sequence of a PCR amplified fragment of human Delta (H-Delta-1) (SEQ ID NO:14) and the predicted samino acid sequences using the three available open reading frames, 2nd line (SEQ ID NO:15), 3rd line (SEQ ID NO:16), 4th line (SEQ ID NO:17).

Figure 11. An alignment of human H-Delta-1 (top line) and chick C-Delta-1 (bottom line). The predicted amino acid 10 sequence of human Delta (SEQ ID NO:18) is shown in the top line. The sequence of human Delta was determined by "eye", in which the sequence of the appropriate reading frame was determined by maximizing homology with C-Delta-1. No single reading frame shown in Figure 10 gave the correct sequence 15 due to errors in the DNA sequence of Figure 10 that caused reading frameshifts.

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Figure 12A-12B. Figure 12A presents the contig DNA sequence of human Delta (H-Delta-1) (SEQ ID NO:33) from clone HDl 18. Figure 12B presents the nucleotide sequence shown in

- 20 Figure 12A (top line, SEQ ID NO:33) and the deduced amino acid sequences using the three possible open reading frames, second line (SEQ ID NO:34), third line (SEQ ID NO:35), fourth line (SEQ ID NO:36). The amino acid sequence with the greatest homology to the mouse Delta-1 amino acid sequence is
- 25 boxed. This boxed amino acid sequence is the predicted amino acid sequence of human Delta; where the reading frame shifts indicates where a sequencing error is present in the sequence. No single reading frame shown in Figure 12A gave an uninterrupted amino acid sequence due to errors in the DNA
- 30 sequence that caused shifts in the reading frame. X indicates an undetermined amino acid; N indicates an undetermined nucleotide.

Figure 13. An alignment of mouse M-Delta-1 DNA sequence (top line, SEQ ID NO:37) and human H-Delta-1 DNA sequence (second line, SEQ ID NO:33) and their consensus sequence (third line, SEQ ID NO:38).

Figure 14. The composite human Delta (H-Delta-1) amino acid sequence (SEQ ID NOS:39-65, respectively) is presented, representing the boxed amino sequence from Figure 12B. ">" indicates that the sequence continues on the line below. "*" indicates a break in the sequence.

5. <u>DETAILED DESCRIPTION OF THE INVENTION</u>

The present invention relates to nucleotide sequences of vertebrate Delta genes, and amino acid sequences of their encoded proteins. The invention further relates to fragments and other derivatives, and analogs, of vertebrate Delta proteins. Nucleic acids encoding such fragments or derivatives are also within the scope of the invention. The invention provides Delta genes and their encoded proteins of many different vertebrate species. The Delta genes of the invention include chick, mouse, and human Delta and related genes (homologs) in other vertebrate species. In specific embodiments, the Delta genes and proteins are from vertebrates, or more particularly, mammals. In a preferred embodiment of the invention, the Delta protein is a human protein. Production of the foregoing proteins and

derivatives, e.g., by recombinant methods, is provided.

The invention relates to Delta derivatives and analogs of the invention which are functionally active, i.e., they are capable of displaying one or more known functional activities associated with a full-length (wild-type) Delta protein. Such functional activities include but are not limited to antigenicity [ability to bind (or compete with Delta for binding) to an anti-Delta antibody], immunogenicity (ability to generate antibody which binds to Delta), ability to bind (or compete with Delta for binding) to Notch or other toporythmic proteins or fragments thereof ("adhesiveness"), ability to bind (or compete with Delta for binding) to a receptor for Delta, ability to affect cell fate

35 differentiation, and therapeutic activity. "Toporythmic

proteins" as used herein, refers to the protein products of



as other members of this interacting gene family which may be identified, e.g., by virtue of the ability of their gene sequences to hybridize, or their homology to Delta, Serrate, or Notch, or the ability of their genes to display phenotypic interactions.

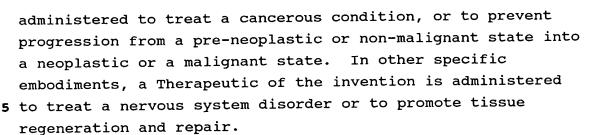
The invention further relates to fragments (and derivatives and analogs thereof) of Delta which comprise one or more domains of the Delta protein, including but not limited to the intracellular domain, extracellular domain,

10 DSL domain, region amino-terminal to the DSL domain, transmembrane domain, membrane-associated region, or one or more EGF-like (homologous) repeats of a Delta protein, or any combination of the foregoing.

Antibodies to vertebrate Delta, its derivatives and 15 analogs, are additionally provided.

As demonstrated infra, Delta plays a critical role in development and other physiological processes, in particular, as a ligand to Notch, which is involved in cell fate (differentiation) determination. In particular, Delta

- 20 is believed to play a major role in determining cell fates in the central nervous system. The nucleic acid and amino acid sequences and antibodies thereto of the invention can be used for the detection and quantitation of Delta mRNA and protein of human and other species, to study expression thereof, to
- 25 produce Delta and fragments and other derivatives and analogs thereof, in the study and manipulation of differentiation and other physiological processes. The present invention also relates to therapeutic and diagnostic methods and compositions based on Delta proteins and nucleic acids. The
- 30 invention provides for treatment of disorders of cell fate or differentiation by administration of a therapeutic compound of the invention. Such therapeutic compounds (termed herein "Therapeutics") include: Delta proteins and analogs and derivatives (including fragments) thereof; antibodies
- 35 thereto; nucleic acids encoding the Delta proteins, analogs, or derivatives; and Delta antisense nucleic acids. In a preferred embodiment, a Therapeutic of the invention is



In one embodiment, Therapeutics which antagonize, or inhibit, Notch and/or Delta function (hereinafter "Antagonist Therapeutics") are administered for therapeutic effect. In another embodiment, Therapeutics which promote Notch and/or Delta function (hereinafter "Agonist Therapeutics") are administered for therapeutic effect.

Disorders of cell fate, in particular hyperproliferative (e.g., cancer) or hypoproliferative

15 disorders, involving aberrant or undesirable levels of expression or activity or localization of Notch and/or Delta protein can be diagnosed by detecting such levels, as described more fully infra.

In a preferred aspect, a Therapeutic of the

20 invention is a protein consisting of at least a fragment

(termed herein "adhesive fragment") of Delta which mediates
binding to a Notch protein or a fragment thereof.

The invention is illustrated by way of examples infra which disclose, inter alia, the cloning of a chick

25 Delta homolog (Section 6), the cloning of a mouse Delta homolog (Section 7), and the cloning of a human Delta homolog (Section 8).

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is 30 divided into the subsections which follow.

5.1. ISOLATION OF THE DELTA GENES

The invention relates to the nucleotide sequences of vertebrate *Delta* nucleic acids. In specific embodiments, 35 human *Delta* nucleic acids comprise the cDNA sequences shown in Figure 10 (SEQ ID NO:14) or in Figure 12A (SEQ ID NO:33), or the coding regions thereof, or nucleic



acids encoding a vertebrate Delta protein (e.g., having the sequence of SEQ ID NO:1, 3, 11, 14 or 33). The invention provides nucleic acids consisting of at least 8 nucleotides (i.e., a hybridizable portion) of a vertebrate Delta

- 5 sequence; in other embodiments, the nucleic acids consist of at least 25 (continuous) nucleotides, 50 nucleotides, 100 nucleotides, 150 nucleotides, or 200 nucleotides of a *Delta* sequence, or a full-length *Delta* coding sequence. The invention also relates to nucleic acids hybridizable to or
- 10 complementary to the foregoing sequences or their complements. In specific aspects, nucleic acids are provided which comprise a sequence complementary to at least 10, 25, 50, 100, or 200 nucleotides or the entire coding region of a vertebrate Delta gene. In a specific embodiment, a nucleic
- 15 acid which is hybridizable to a vertebrate (e.g., mammalian)

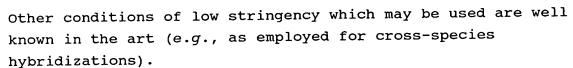
 Delta nucleic acid (e.g., having sequence SEQ ID NO:14 or SEQ

 ID NO:33, or an at least 10, 25, 50, 100, or 200 nucleotide

 portion thereof), or to a nucleic acid encoding a Delta

 derivative, under conditions of low stringency is provided.
- 20 By way of example and not limitation, procedures using such conditions of low stringency are as follows (see also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792): Filters containing DNA are pretreated for 6 h at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl
- 25 (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μ g/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μ g/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 106 cpm
- 30 ³²P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an
- 35 additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film.

20 known in the art.



In another specific embodiment, a nucleic acid

5 which is hybridizable to a vertebrate (e.g., mammalian) Delta nucleic acid under conditions of high stringency is provided. By way of example and not limitation, procedures using such conditions of high stringency are as follows:

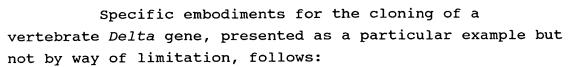
Prehybridization of filters containing DNA is carried out for

10 8 h to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 μ g/ml denatured salmon sperm DNA. Filters are hybridized for 48 h at 65°C in prehybridization mixture containing 100 μ g/ml denatured salmon sperm DNA and 5-20 X 106

15 cpm of ³²P-labeled probe. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 min before autoradiography. Other conditions of high stringency which may be used are well

Nucleic acids encoding fragments and derivatives of vertebrate Delta proteins (see Section 5.6), and Delta antisense nucleic acids (see Section 5.11) are additionally provided. As is readily apparent, as used herein, a "nucleic acid encoding a fragment or portion of a Delta protein" shall be construed as referring to a nucleic acid encoding only the recited fragment or portion of the Delta protein and not the other contiguous portions of the Delta protein as a continuous sequence.

Fragments of vertebrate Delta nucleic acids
comprising regions of homology to other toporythmic proteins
are also provided. The DSL regions (regions of homology with
Drosophila Serrate and Delta) of Delta proteins of other
species are also provided. Nucleic acids encoding conserved
regions between Delta and Serrate, such as those shown in
Figures 3 and 8 are also provided.

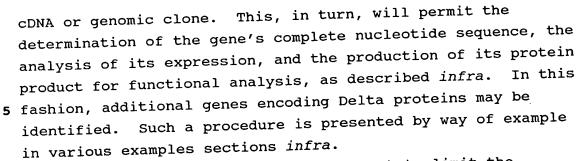


For expression cloning (a technique commonly known 5 in the art), an expression library is constructed by methods known in the art. For example, mRNA (e.g., human) is isolated, cDNA is made and ligated into an expression vector (e.g., a bacteriophage derivative) such that it is capable of being expressed by the host cell into which it is then

10 introduced. Various screening assays can then be used to select for the expressed Delta product. In one embodiment, anti-Delta antibodies can be used for selection.

In another preferred aspect, PCR is used to amplify the desired sequence in a genomic or cDNA library, prior to selection. Oligonucleotide primers representing known Delta sequences (preferably vertebrate sequences) can be used as primers in PCR. In a preferred aspect, the oligonucleotide primers represent at least part of the Delta conserved segments of strong homology between Serrate and Delta. The

- 20 synthetic oligonucleotides may be utilized as primers to amplify by PCR sequences from a source (RNA or DNA), preferably a cDNA library, of potential interest. PCR can be carried out, e.g., by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp"). The DNA being
- 25 amplified can include mRNA or cDNA or genomic DNA from any eukaryotic species. One can choose to synthesize several different degenerate primers, for use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for
- 30 greater or lesser degrees of nucleotide sequence similarity between the known Delta nucleotide sequence and the nucleic acid homolog being isolated. For cross species hybridization, low stringency conditions are preferred. For same species hybridization, moderately stringent conditions
- 35 are preferred. After successful amplification of a segment of a *Delta* homolog, that segment may be molecularly cloned and sequenced, and utilized as a probe to isolate a complete



The above-methods are not meant to limit the following general description of methods by which clones of 10 Delta may be obtained.

Any vertebrate cell potentially can serve as the nucleic acid source for the molecular cloning of the *Delta* gene. The nucleic acid sequences encoding Delta can be isolated from mammalian, human, porcine, bovine, feline,

- 15 avian, equine, canine, as well as additional primate sources, etc. For example, we have amplified fragments of the Delta gene in mouse, chicken, and human, by PCR using cDNA libraries with Delta primers. The DNA may be obtained by standard procedures known in the art from cloned DNA (e.g., a
- 20 DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell. (See, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York;
- 25 Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.) Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source,
- 30 the gene should be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will encode the desired gene. The DNA may be cleaved at specific sites

35 using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by



sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

- Once the DNA fragments are generated, identification of the specific DNA fragment containing the desired gene may be accomplished in a number of ways. For example, if an amount of a portion of a Delta (of any species) gene or its specific RNA, or a fragment thereof,
- 10 e.g., an extracellular domain (see Section 5.6), is available and can be purified and labeled, the generated DNA fragments may be screened by nucleic acid hybridization to the labeled probe (Benton, W. and Davis, R., 1977, Science 196:180; Grunstein, M. And Hogness, D., 1975, Proc. Natl. Acad. Sci.
- 15 U.S.A. 72:3961). Those DNA fragments with substantial homology to the probe will hybridize. It is also possible to identify the appropriate fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such is
- 20 available. Further selection can be carried out on the basis of the properties of the gene. Alternatively, the presence of the gene may be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For example, cDNA clones, or DNA clones which
- 25 hybrid-select the proper mRNAs, can be selected which produce a protein that, e.g., has similar or identical electrophoretic migration, isolectric focusing behavior, proteolytic digestion maps, binding activity, in vitro aggregation activity ("adhesiveness") or antigenic properties
- 30 as known for Delta. If an antibody to Delta is available, the Delta protein may be identified by binding of labeled antibody to the putatively Delta synthesizing clones, in an ELISA (enzyme-linked immunosorbent assay)-type procedure.

The Delta gene can also be identified by mRNA

35 selection by nucleic acid hybridization followed by in vitro translation. In this procedure, fragments are used to isolate complementary mRNAs by hybridization. Such DNA



fragments may represent available, purified *Delta* DNA of another species (e.g., *Drosophila*). Immunoprecipitation analysis or functional assays (e.g., aggregation ability in vitro; binding to receptor; see *infra*) of the *in vitro*

- 5 translation products of the isolated products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments that contain the desired sequences. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies
- 10 specifically directed against Delta protein. A radiolabelled Delta cDNA can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabelled mRNA or cDNA may then be used as a probe to identify the Delta DNA fragments from among other genomic DNA fragments.
- include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes the Delta protein. For example, RNA for cDNA cloning of the Delta gene can be isolated from cells which express Delta. Other methods are possible and within

The identified and isolated gene can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used.

- 25 Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as PBR322 or pUC plasmid derivatives. The
- 30 insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA
- 35 molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may



comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and *Delta* gene may be modified by homopolymeric tailing. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated.

In an alternative method, the desired gene may be identified and isolated after insertion into a suitable 10 cloning vector in a "shot gun" approach. Enrichment for the desired gene, for example, by size fractionation, can be done before insertion into the cloning vector.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate the isolated Delta gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

The Delta sequences provided by the instant invention include those nucleotide sequences encoding substantially the same amino acid sequences as found in native vertebrate Delta proteins, and those encoded amino acid sequences with functionally equivalent amino acids, all as described in Section 5.6 infra for Delta derivatives.

5.2. EXPRESSION OF THE DELTA GENES

The nucleotide sequence coding for a vertebrate

30 Delta protein or a functionally active fragment or other derivative thereof (see Section 5.6), can be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary

35 transcriptional and translational signals can also be

stranscriptional and translational signals can also be supplied by the native *Delta* gene and/or its flanking regions. A variety of host-vector systems may be utilized to





express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such 5 as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation 10 elements may be used. In a specific embodiment, the adhesive portion of the Delta gene is expressed. In other specific embodiments, the human Delta gene is expressed, or a sequence encoding a functionally active portion of human Delta. yet another embodiment, a fragment of Delta comprising the 15 extracellular domain, or other derivative, or analog of Delta is expressed.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene 20 consisting of appropriate transcriptional/translational control signals and the protein coding sequences. methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinants (genetic recombination). Expression of nucleic acid sequence encoding a Delta protein 25 or peptide fragment may be regulated by a second nucleic acid sequence so that the Delta protein or peptide is expressed in a host transformed with the recombinant DNA molecule. example, expression of a Delta protein may be controlled by any promoter/enhancer element known in the art. Promoters 30 which may be used to control Delta gene expression include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the 35 herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982,



Nature 296:39-42); prokaryotic expression vectors such as the β -lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-

- 5 25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner, et al.,
- 10 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK
- 15 (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et
- 20 al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is
- 25 active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al.,
- 30 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-
- 35 antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), betaglobin gene control region which is active in myeloid cells



(Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-5 2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the

hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Expression vectors containing Delta gene inserts

- 10 can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by nucleic acid
- 15 hybridization using probes comprising sequences that are homologous to an inserted toporythmic gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase
- 20 activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the Delta gene is inserted within the marker gene sequence of the vector, recombinants containing the Delta
- 25 insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties
- 30 of the *Delta* gene product *in vitro* assay systems, e.g., aggregation (binding) with Notch, binding to a receptor, binding with antibody.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may 35 be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As



previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (e.g., lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered Delta protein may be controlled. Furthermore, different host cells have

- 15 characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, cleavage [e.g., of signal sequence]) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of
- 20 the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of a heterologous
- 25 mammalian Delta protein. Furthermore, different vector/host expression systems may effect processing reactions such as proteolytic cleavages to different extents.

In other specific embodiments, the Delta protein, fragment, analog, or derivative may be expressed as a fusion, or chimeric protein product (comprising the protein, fragment, analog, or derivative joined via a peptide bond to a heterologous protein sequence (of a different protein)). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino

35 acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a

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chimeric product may be made by protein synthetic techniques, e.g., by use of a peptide synthesizer.

Both cDNA and genomic sequences can be cloned and expressed.

5.3. IDENTIFICATION AND PURIFICATION OF THE DELTA GENE PRODUCTS

In particular aspects, the invention provides amino acid sequences of a vertebrate Delta, preferably a human Delta, and fragments and derivatives thereof which comprise an antigenic determinant (i.e., can be recognized by an antibody) or which are otherwise functionally active, as well as nucleic acid sequences encoding the foregoing.

"Functionally active" material as used herein refers to that material displaying one or more known functional activities associated with a full-length (wild-type) Delta protein, e.g., binding to Notch or a portion thereof, binding to any other Delta ligand, antigenicity (binding to an anti-Delta antibody), etc.

In specific embodiments, the invention provides
fragments of a Delta protein consisting of at least 6 amino
acids, 10 amino acids, 25 amino acids, 50 amino acids, or of
at least 75 amino acids. Molecules comprising such fragments
are also provided. In other embodiments, the proteins
comprise or consist essentially of an extracellular domain,
DSL domain, epidermal growth factor-like repeat (ELR) domain,
one or any combination of ELRs, transmembrane domain, or
intracellular (cytoplasmic) domain, or a portion which binds
to Notch, or any combination of the foregoing, of a
vertebrate Delta protein. Fragments, or proteins comprising
fragments, lacking some or all of the foregoing regions of a
Delta protein are also provided. Nucleic acids encoding the
foregoing are provided.

Once a recombinant which expresses the *Delta* gene sequence is identified, the gene product can be analyzed.

This is achieved by assays based on the physical or functional properties of the product, including radioactive

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labelling of the product followed by analysis by gel electrophoresis, immunoassay, etc.

Once the Delta protein is identified, it may be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. The functional properties may be evaluated using any suitable assay (see Section 5.7).

10 Alternatively, once a Delta protein produced by a recombinant is identified, the amino acid sequence of the protein can be deduced from the nucleotide sequence of the chimeric gene contained in the recombinant. As a result, the protein can be synthesized by standard chemical methods known 15 in the art (e.g., see Hunkapiller, M., et al., 1984, Nature 310:105-111).

In a specific embodiment of the present invention, such Delta proteins, whether produced by recombinant DNA techniques or by chemical synthetic methods, include but are not limited to those containing, as a primary amino acid sequence, all or part of the amino acid sequences substantially as depicted in Figures 2, 8, 11 or 14 (SEQ ID NOS:2, 10, 16 and 39-65), as well as fragments and other derivatives, and analogs thereof.

5.4. STRUCTURE OF THE DELTA GENES AND PROTEINS

The structure of the vertebrate Delta genes and proteins can be analyzed by various methods known in the art.

5.4.1. GENETIC ANALYSIS

The cloned DNA or cDNA corresponding to the *Delta* gene can be analyzed by methods including but not limited to Southern hybridization (Southern, E.M., 1975, J. Mol. Biol. 98:503-517), Northern hybridization (see e.g., Freeman et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:4094-4098), restriction endonuclease mapping (Maniatis, T., 1982, Molecular Cloning, A Laboratory, Cold Spring Harbor, New

York), and DNA sequence analysis. Polymerase chain reaction (PCR; U.S. Patent Nos. 4,683,202, 4,683,195 and 4,889,818; Gyllenstein et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7652-7656; Ochman et al., 1988, Genetics 120:621-623; Loh

- 5 et al., 1989, Science 243:217-220) followed by Southern hybridization with a *Delta*-specific probe can allow the detection of the *Delta* gene in DNA from various cell types. Methods of amplification other than PCR are commonly known and can also be employed. In one embodiment, Southern
- 10 hybridization can be used to determine the genetic linkage of Delta. Northern hybridization analysis can be used to determine the expression of the Delta gene. Various cell types, at various states of development or activity can be tested for Delta expression. Examples of such techniques and
- 15 their results are described in Section 6, infra. The stringency of the hybridization conditions for both Southern and Northern hybridization can be manipulated to ensure detection of nucleic acids with the desired degree of relatedness to the specific Delta probe used.
- Restriction endonuclease mapping can be used to roughly determine the genetic structure of the *Delta* gene.

 Restriction maps derived by restriction endonuclease cleavage can be confirmed by DNA sequence analysis.

DNA sequence analysis can be performed by any

25 techniques known in the art, including but not limited to the method of Maxam and Gilbert (1980, Meth. Enzymol. 65:499-560), the Sanger dideoxy method (Sanger, F., et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5463), the use of T7 DNA polymerase (Tabor and Richardson, U.S. Patent No. 4,795,699),

30 or use of an automated DNA sequenator (e.g., Applied Biosystems, Foster City, CA).

5.4.2. PROTEIN ANALYSIS

The amino acid sequence of the Delta protein can be 35 derived by deduction from the DNA sequence, or alternatively, by direct sequencing of the protein, e.g., with an automated amino acid sequencer. The amino acid sequence of a

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representative Delta protein comprises the sequence substantially as depicted in Figure 2, and detailed in Section 6, *infra*, with the representative mature protein that shown by amino acid numbers 1-728.

The Delta protein sequence can be further characterized by a hydrophilicity analysis (Hopp, T. and Woods, K., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:3824). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic regions of the Delta protein and the corresponding regions of the gene sequence which encode such regions. Hydrophilic regions are more likely to be immunogenic.

Secondary, structural analysis (Chou, P. and Fasman, G., 1974, Biochemistry 13:222) can also be done, to 15 identify regions of Delta that assume specific secondary structures.

Manipulation, translation, and secondary structure prediction, as well as open reading frame prediction and plotting, can also be accomplished using computer software 20 programs available in the art.

Other methods of structural analysis can also be employed. These include but are not limited to X-ray crystallography (Engstom, A., 1974, Biochem. Exp. Biol. 11:7-13) and computer modeling (Fletterick, R. and Zoller, M.

25 (eds.), 1986, Computer Graphics and Molecular Modeling, in Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

5.5. GENERATION OF ANTIBODIES TO DELTA PROTEINS AND DERIVATIVES THEREOF

According to the invention, a vertebrate Delta protein, its fragments or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which recognize such an immunogen. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. In a specific embodiment, antibodies to human Delta are

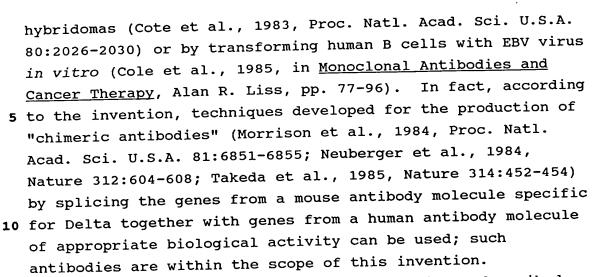
produced. In another embodiment, antibodies to the extracellular domain of Delta are produced. In another embodiment, antibodies to the intracellular domain of Delta are produced.

Various procedures known in the art may be used for the production of polyclonal antibodies to a Delta protein or derivative or analog. In a particular embodiment, rabbit polyclonal antibodies to an epitope of the Delta protein encoded by a sequence depicted in Figures 1a, 1b, 7 or 11, or

- of antibody, various host animals can be immunized by injection with the native Delta protein, or a synthetic version, or derivative (e.g., fragment) thereof, including but not limited to rabbits, mice, rats, etc. Various
- 15 adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil
- 20 emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum

For preparation of monoclonal antibodies directed toward a Delta protein sequence or analog thereof, any

- 25 technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique
- 30 (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be
- 35 produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human



According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 15 4,946,778) can be adapted to produce Delta-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for Delta

proteins, derivatives, or analogs.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the

25 F(ab')₂ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing 30 agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, e.g. ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize a specific

35 domain of a vertebrate Delta protein, one may assay generated hybridomas for a product which binds to a Delta fragment containing such domain. For selection of an antibody





immunospecific to human Delta, one can select on the basis of positive binding to human Delta and a lack of binding to Drosophila Delta.

The foregoing antibodies can be used in methods

5 known in the art relating to the localization and activity of
the protein sequences of the invention (e.g., see Section
5.7, infra), e.g., for imaging these proteins, measuring
levels thereof in appropriate physiological samples, in
diagnostic methods, etc.

Antibodies specific to a domain of a Delta protein are also provided. In a specific embodiment, antibodies which bind to a Notch-binding fragment of Delta are provided.

In another embodiment of the invention (see infra), anti-Delta antibodies and fragments thereof containing the 15 binding domain are Therapeutics.

5.6. DELTA PROTEINS, DERIVATIVES AND ANALOGS

The invention further relates to vertebrate (e.g., mammalian) Delta proteins, and derivatives (including but not limited to fragments) and analogs of vertebrate Delta proteins. Nucleic acids encoding Delta protein derivatives and protein analogs are also provided. In one embodiment, the Delta proteins are encoded by the Delta nucleic acids described in Section 5.1 supra. In particular aspects, the proteins, derivatives, or analogs are of mouse, chicken, rat, pig, cow, dog, monkey, or human Delta proteins. In a specific embodiment, a mature, full-length vertebrate Delta protein is provided. In one embodiment, a vertebrate Delta protein lacking only the signal sequence (approximately the

The production and use of derivatives and analogs related to Delta are within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, i.e., capable of exhibiting one or more functional activities associated with a full-length, wild-type Delta protein. As one example, such derivatives or analogs which have the desired immunogenicity



or antigenicity can be used, for example, in immunoassays, for immunization, for inhibition of Delta activity, etc. Such molecules which retain, or alternatively inhibit, a desired Delta property, e.g., binding to Notch or other

- 5 toporythmic proteins, binding to a cell-surface receptor, can be used as inducers, or inhibitors, respectively, of such property and its physiological correlates. A specific embodiment relates to a Delta fragment that can be bound by an anti-Delta antibody but cannot bind to a Notch protein or
- 10 other toporythmic protein. Derivatives or analogs of Delta can be tested for the desired activity by procedures known in the art, including but not limited to the assays described in Section 5.7.

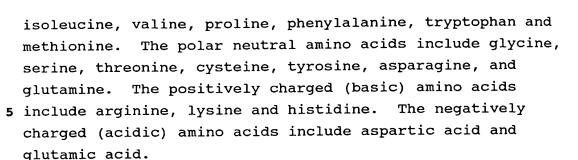
In particular, Delta derivatives can be made by

15 altering Delta sequences by substitutions, additions or
deletions that provide for functionally equivalent molecules.

Due to the degeneracy of nucleotide coding sequences, other

DNA sequences which encode substantially the same amino acid
sequence as a Delta gene may be used in the practice of the

- 20 present invention. These include but are not limited to nucleotide sequences comprising all or portions of Delta genes which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change.
- 25 Likewise, the Delta derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a Delta protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues
- 30 within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within
- 35 the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine,



In a specific embodiment of the invention, proteins consisting of or comprising a fragment of a vertebrate Delta protein consisting of at least 10 (continuous) amino acids of the Delta protein is provided. In other embodiments, the fragment consists of at least 20 or 50 amino acids of the Delta protein. In specific embodiments, such fragments are not larger than 35, 100 or 200 amino acids. Derivatives or 15 analogs of Delta include but are not limited to those peptides which are substantially homologous to a vertebrate Delta protein or fragments thereof (e.g., at least 30%, 50%, 70%, or 90% identity over an amino acid sequence of identical size -- e.g., comprising a domain) or whose encoding nucleic 20 acid is capable of hybridizing to a coding Delta sequence.

The Delta derivatives and analogs of the invention

can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned Delta

25 gene sequence can be modified by any of numerous strategies known in the art (Maniatis, T., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s),

30 followed by further enzymatic modification if desired, isolated, and ligated in vitro. In the production of the gene encoding a derivative or analog of Delta, care should be taken to ensure that the modified gene remains within the same translational reading frame as Delta, uninterrupted by translational stop signals, in the gene region where the

desired Delta activity is encoded.



Additionally, the Delta-encoding nucleic acid sequence can be mutated in vitro or in vivo, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or

- 5 form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* sitedirected mutagenesis (Hutchinson, C., et al., 1978, J. Biol.
- 10 Chem 253:6551), use of TAB® linkers (Pharmacia), etc. PCR primers containing sequence changes can be used in PCR to introduce such changes into the amplified fragments.

Manipulations of the Delta sequence may also be made at the protein level. Included within the scope of the invention are Delta protein fragments or other derivatives or analogs which are differentially modified during or after translation, e.g., by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to

- 20 an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄; acetylation, formylation, oxidation,
- 25 reduction; metabolic synthesis in the presence of tunicamycin; etc.

In addition, analogs and derivatives of Delta can be chemically synthesized. For example, a peptide corresponding to a portion of a Delta protein which comprises

- 30 the desired domain (see Section 5.6.1), or which mediates the desired aggregation activity in vitro, or binding to a receptor, can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or
- 35 addition into the Delta sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α -amino isobutyric acid, 4-aminobutyric acid,





hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -alanine, designer amino acids such as β -methyl amino acids, C α -methyl amino acids, and N α -methyl

5 amino acids. In a specific embodiment, the Delta derivative is a chimeric, or fusion, protein comprising a vertebrate Delta protein or fragment thereof (preferably consisting of at least a domain or motif of the Delta protein, or at least 10 10 amino acids of the Delta protein) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. In one embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid encoding the protein (comprising a Delta-coding sequence 15 joined in-frame to a coding sequence for a different protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric 20 product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, e.g., by use of a peptide synthesizer. specific embodiment, a chimeric nucleic acid encoding a mature Delta protein with a heterologous signal sequence is 25 expressed such that the chimeric protein is expressed and processed by the cell to the mature Delta protein. another example, and not by way of limitation, a recombinant molecule can be constructed according to the invention,

- comprising coding portions of both *Delta* and another

 30 toporythmic gene, e.g., Serrate. The encoded protein of such a recombinant molecule could exhibit properties associated with both Serrate and Delta and portray a novel profile of biological activities, including agonists as well as antagonists. The primary sequence of Delta and Serrate may
- 35 also be used to predict tertiary structure of the molecules using computer simulation (Hopp and Woods, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:3824-3828); Delta/Serrate chimeric

25

recombinant genes could be designed in light of correlations between tertiary structure and biological function. Likewise, chimeric genes comprising portions of Delta fused to any heterologous protein-encoding sequences may be 5 constructed. A specific embodiment relates to a chimeric protein comprising a fragment of Delta of at least six amino acids.

In another specific embodiment, the Delta derivative is a fragment of vertebrate Delta comprising a 10 region of homology with another toporythmic protein. herein, a region of a first protein shall be considered "homologous" to a second protein when the amino acid sequence of the region is at least 30% identical or at least 75% either identical or involving conservative changes, when 15 compared to any sequence in the second protein of an equal number of amino acids as the number contained in the region. For example, such a Delta fragment can comprise one or more regions homologous to Serrate, including but not limited to the DSL domain or a portion thereof.

Other specific embodiments of derivatives and 20 analogs are described in the subsections below and examples sections infra.

5.6.1. DERIVATIVES OF DELTA CONTAINING ONE OR MORE DOMAINS OF THE PROTEIN

In a specific embodiment, the invention relates to vertebrate Delta derivatives and analogs, in particular Delta fragments and derivatives of such fragments, that comprise, or alternatively consist of, one or more domains of the Delta 30 protein, including but not limited to the extracellular domain, signal sequence, region amino-terminal to the DSL domain, DSL domain, ELR domain, transmembrane domain, intracellular domain, and one or more of the EGF-like repeats (ELR) of the Delta protein (e.g., ELRs 1-9), or any combination of the foregoing. In particular examples relating to the chick and mouse Delta proteins, such domains are identified in Examples Section 6 and 7, respectively, and





in Figures 3 and 9. Thus, by way of example is provided, a molecule comprising an extracellular domain (approximately amino acids 1-545), signal sequence (approximately amino acids 1-17), region amino-terminal to the DSL domain

- 5 (approximately amino acids 1-178), the DSL domain (approximately amino acids 179-223), EGF1 (approximately amino acids 229-260), EGF2 (approximately amino acids 261-292), EGF3 (approximately amino acids 293-332), EGF4 (approximately amino acids 333-370), EGF5 (approximately
- 10 amino acids 371-409), EGF6 (approximately amino acids 410-447), EGF7 (approximately amino acids 448-485), EGF8 (approximately amino acids 486-523), transmembrane domain, and intracellular (cytoplasmic) domain (approximately amino acids 555-728) of a vertebrate Delta.
- In a specific embodiment, the molecules comprising specific fragments of vertebrate Delta are those comprising fragments in the respective Delta protein most homologous to specific fragments of the *Drosophila* or chick Delta protein. In particular embodiments, such a molecule comprises or
- 20 consists of the amino acid sequences of SEQ ID NO:2 or 16. Alternatively, a fragment comprising a domain of a Delta homolog can be identified by protein analysis methods as described in Section 5.3.2.

5.6.2. DERIVATIVES OF DELTA THAT MEDIATE BINDING TO TOPORYTHMIC PROTEIN DOMAINS

The invention also provides for vertebrate Delta fragments, and analogs or derivatives of such fragments, which mediate binding to toporythmic proteins (and thus are termed herein "adhesive"), and nucleic acid sequences encoding the foregoing.

In a particular embodiment, the adhesive fragment of a Delta protein comprises the DSL domain, or a portion thereof. Subfragments within the DSL domain that mediate binding to Notch can be identified by analysis of constructs expressing deletion mutants.

The ability to bind to a toporythmic protein (preferably Notch) can be demonstrated by in vitro aggregation assays with cells expressing such a toporythmic protein as well as cells expressing Delta or a Delta derivative (See Section 5.7). That is, the ability of a Delta fragment to bind to a Notch protein can be demonstrated by detecting the ability of the Delta fragment, when expressed on the surface of a first cell, to bind to a Notch protein expressed on the surface of a second cell.

The nucleic acid sequences encoding toporythmic proteins or adhesive domains thereof, for use in such assays, can be isolated from human, porcine, bovine, feline, avian, equine, canine, or insect, as well as primate sources and any other species in which homologs of known toporythmic genes

15 can be identified.

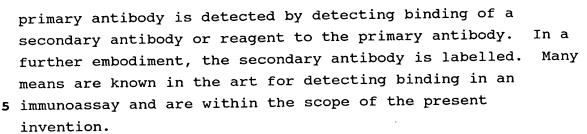
5.7. ASSAYS OF DELTA PROTEINS, DERIVATIVES AND ANALOGS

The functional activity of vertebrate Delta

20 proteins, derivatives and analogs can be assayed by various methods.

For example, in one embodiment, where one is assaying for the ability to bind or compete with wild-type Delta for binding to anti-Delta antibody, various

- immunoassays known in the art can be used, including but not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions,
- immunodiffusion assays, in situ immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays,
- protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the



In another embodiment, where one is assaying for the ability to mediate binding to a toporythmic protein, e.g., Notch, one can carry out an *in vitro* aggregation assay 10 (see Fehon et al., 1990, Cell 61:523-534; Rebay et al., 1991, Cell 67:687-699).

In another embodiment, where a receptor for Delta is identified, receptor binding can be assayed, e.g., by means well-known in the art. In another embodiment,

15 physiological correlates of Delta binding to cells expressing a Delta receptor (signal transduction) can be assayed.

In another embodiment, in insect or other model systems, genetic studies can be done to study the phenotypic effect of a Delta mutant that is a derivative or analog of 20 wild-type Delta.

Other methods will be known to the skilled artisan and are within the scope of the invention.

5.8. THERAPEUTIC USES

of cell fate or differentiation by administration of a therapeutic compound of the invention. Such therapeutic compounds (termed herein "Therapeutics") include: Delta proteins and analogs and derivatives (including fragments)

thereof (e.g., as described hereinabove); antibodies thereto (as described hereinabove); nucleic acids encoding the Delta proteins, analogs, or derivatives (e.g., as described hereinabove); and Delta antisense nucleic acids. As stated supra, the Antagonist Therapeutics of the invention are those

Therapeutics which antagonize, or inhibit, a Delta function and/or Notch function (since Delta is a Notch ligand). Such Antagonist Therapeutics are most preferably identified by use



of known convenient in vitro assays, e.g., based on their ability to inhibit binding of Delta to another protein (e.g., a Notch protein), or inhibit any known Notch or Delta function as preferably assayed in vitro or in cell culture,

- 5 although genetic assays (e.g., in Drosophila) may also be employed. In a preferred embodiment, the Antagonist Therapeutic is a protein or derivative thereof comprising a functionally active fragment such as a fragment of Delta which mediates binding to Notch, or an antibody thereto. In
- 10 other specific embodiments, such an Antagonist Therapeutic is a nucleic acid capable of expressing a molecule comprising a fragment of Delta which binds to Notch, or a *Delta* antisense nucleic acid (see Section 5.11 herein). It should be noted that preferably, suitable in vitro or in vivo assays, as
- 15 described *infra*, should be utilized to determine the effect of a specific Therapeutic and whether its administration is indicated for treatment of the affected tissue, since the developmental history of the tissue may determine whether an Antagonist or Agonist Therapeutic is desired.
- In addition, the mode of administration, e.g., whether administered in soluble form or administered via its encoding nucleic acid for intracellular recombinant expression, of the Delta protein or derivative can affect whether it acts as an agonist or antagonist.
- In another embodiment of the invention, a nucleic acid containing a portion of a *Delta* gene is used, as an Antagonist Therapeutic, to promote *Delta* inactivation by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

The Agonist Therapeutics of the invention, as described *supra*, promote Delta function. Such Agonist Therapeutics include but are not limited to proteins and derivatives comprising the portions of Notch that mediate binding to Delta, and nucleic acids encoding the foregoing (which can be administered to express their encoded products in vivo).



Further descriptions and sources of Therapeutics of the inventions are found in Sections 5.1 through 5.7 herein.

Molecules which retain, or alternatively inhibit, a desired Delta property, e.g., binding to Notch, binding to an intracellular ligand, can be used therapeutically as inducers, or inhibitors, respectively, of such property and its physiological correlates. In a specific embodiment, a peptide (e.g., in the range of 6-50 or 15-25 amino acids; and particularly of about 10, 15, 20 or 25 amino acids)

- 10 containing the sequence of a portion of Delta which binds to Notch is used to antagonize Notch function. In a specific embodiment, such an Antagonist Therapeutic is used to treat or prevent human or other malignancies associated with increased Notch expression (e.g., cervical cancer, colon
- 15 cancer, breast cancer, squamous adenocarcimas (see infra)).

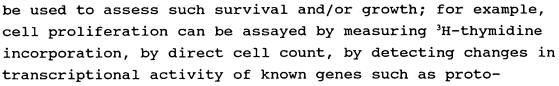
 Derivatives or analogs of Delta can be tested for the desired activity by procedures known in the art, including but not limited to the assays described in the examples infra. For example, molecules comprising Delta fragments which bind to
- 20 Notch EGF-repeats (ELR) 11 and 12 and which are smaller than a DSL domain, can be obtained and selected by expressing deletion mutants and assaying for binding of the expressed product to Notch by any of the several methods (e.g., in vitro cell aggregation assays, interaction trap system), some
- of which are described in the Examples Sections infra. In one specific embodiment, peptide libraries can be screened to select a peptide with the desired activity; such screening can be carried out by assaying, e.g., for binding to Notch or a molecule containing the Notch ELR 11 and 12 repeats.
- other Therapeutics include molecules that bind to a vertebrate Delta protein. Thus, the invention also provides a method for identifying such molecules. Such molecules can be identified by a method comprising contacting a plurality of molecules (e.g., in a peptide library, or combinatorial
- 35 chemical library) with the Delta protein under conditions conducive to binding, and recovering any molecules that bind to the Delta protein.





The Agonist and Antagonist Therapeutics of the invention have therapeutic utility for disorders of cell fate. The Agonist Therapeutics are administered therapeutically (including prophylactically): (1) in diseases or disorders involving an absence or decreased (relative to normal, or desired) levels of Notch or Delta function, for example, in patients where Notch or Delta protein is lacking, genetically defective, biologically inactive or underactive, or underexpressed; and (2) in diseases or disorders wherein in vitro (or in vivo) assays (see infra) indicate the utility of Delta agonist administration. The absence or decreased levels in Notch or Delta function can be readily detected, e.g., by obtaining a patient tissue sample (e.g., from biopsy tissue) and assaying it in vitro for protein levels,

- protein. Many methods standard in the art can be thus employed, including but not limited to immunoassays to detect and/or visualize Notch or Delta protein (e.g., Western blot, immunoprecipitation followed by sodium dodecyl sulfate
- 20 polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect Notch or Delta expression by detecting and/or visualizing respectively Notch or Delta mRNA (e.g., Northern assays, dot blots, in situ hybridization, etc.)
- whether administration of a specific Agonist Therapeutic or Antagonist Therapeutic is indicated, include in vitro cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise administered a
- Therapeutic, and the effect of such Therapeutic upon the tissue sample is observed. In one embodiment, where the patient has a malignancy, a sample of cells from such malignancy is plated out or grown in culture, and the cells are then exposed to a Therapeutic. A Therapeutic which
- 35 inhibits survival or growth of the malignant cells (e.g., by promoting terminal differentiation) is selected for therapeutic use in vivo. Many assays standard in the art can



- 5 oncogenes (e.g., fos, myc) or cell cycle markers; cell viability can be assessed by trypan blue staining, differentiation can be assessed visually based on changes in morphology, etc. In a specific aspect, the malignant cell cultures are separately exposed to (1) an Agonist
- 10 Therapeutic, and (2) an Antagonist Therapeutic; the result of the assay can indicate which type of Therapeutic has therapeutic efficacy.

In another embodiment, a Therapeutic is indicated for use which exhibits the desired effect, inhibition or 15 promotion of cell growth, upon a patient cell sample from tissue having or suspected of having a hyper- or hypoproliferative disorder, respectively. Such hyper- or hypoproliferative disorders include but are not limited to those described in Sections 5.8.1 through 5.8.3 infra.

In another specific embodiment, a Therapeutic is indicated for use in treating nerve injury or a nervous system degenerative disorder (see Section 5.8.2) which exhibits in vitro promotion of nerve regeneration/neurite extension from nerve cells of the affected patient type.

In addition, administration of an Antagonist
Therapeutic of the invention is also indicated in diseases or
disorders determined or known to involve a Notch or Delta
dominant activated phenotype ("gain of function" mutations.)
Administration of an Agonist Therapeutic is indicated in

- 30 diseases or disorders determined or known to involve a Notch or Delta dominant negative phenotype ("loss of function" mutations). The functions of various structural domains of the Notch protein have been investigated in vivo, by ectopically expressing a series of Drosophila Notch deletion
- 35 mutants under the hsp70 heat-shock promoter, as well as eye-specific promoters (see Rebay et al., 1993, Cell 74:319-329).
 Two classes of dominant phenotypes were observed, one

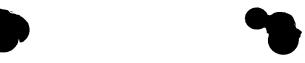


suggestive of Notch loss-of function mutations and the other of Notch gain-of-function mutations. Dominant "activated" phenotypes resulted from overexpression of a protein lacking most extracellular sequences, while dominant "negative"

- 5 phenotypes resulted from overexpression of a protein lacking most intracellular sequences. The results indicated that Notch functions as a receptor whose extracellular domain mediates ligand-binding, resulting in the transmission of developmental signals by the cytoplasmic domain.
- In various specific embodiments, in vitro assays can be carried out with representative cells of cell types involved in a patient's disorder, to determine if a Therapeutic has a desired effect upon such cell types.
- In another embodiment, cells of a patient tissue

 15 sample suspected of being pre-neoplastic are similarly plated out or grown in vitro, and exposed to a Therapeutic. The Therapeutic which results in a cell phenotype that is more normal (i.e., less representative of a pre-neoplastic state, neoplastic state, malignant state, or transformed phenotype)
- 20 is selected for therapeutic use. Many assays standard in the art can be used to assess whether a pre-neoplastic state, neoplastic state, or a transformed or malignant phenotype, is present. For example, characteristics associated with a transformed phenotype (a set of in vitro characteristics
- 25 associated with a tumorigenic ability in vivo) include a more rounded cell morphology, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, release of proteases such as plasminogen activator, increased sugar transport, decreased serum requirement, expression of fetal
- 30 antigens, disappearance of the 250,000 dalton surface protein, etc. (see Luria et al., 1978, General Virology, 3d Ed., John Wiley & Sons, New York pp. 436-446).

In other specific embodiments, the in vitro assays described supra can be carried out using a cell line, rather than a cell sample derived from the specific patient to be treated, in which the cell line is derived from or displays characteristic(s) associated with the malignant, neoplastic



or pre-neoplastic disorder desired to be treated or prevented, or is derived from the neural or other cell type upon which an effect is desired, according to the present invention.

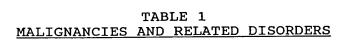
- The Antagonist Therapeutics are administered therapeutically (including prophylactically): (1) in diseases or disorders involving increased (relative to normal, or desired) levels of Notch or Delta function, for example, where the Notch or Delta protein is overexpressed or
- 10 overactive; and (2) in diseases or disorders wherein in vitro (or in vivo) assays indicate the utility of Delta antagonist administration. The increased levels of Notch or Delta function can be readily detected by methods such as those described above, by quantifying protein and/or RNA. In vitro
- 15 assays with cells of patient tissue sample or the appropriate cell line or cell type, to determine therapeutic utility, can be carried out as described above.

5.8.1. MALIGNANCIES

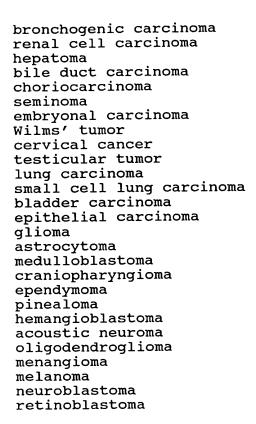
Malignant and pre-neoplastic conditions which can be tested as described *supra* for efficacy of intervention with Antagonist or Agonist Therapeutics, and which can be treated upon thus observing an indication of therapeutic utility, include but are not limited to those described below 25 in Sections 5.8.1 and 5.9.1.

Malignancies and related disorders, cells of which type can be tested *in vitro* (and/or *in vivo*), and upon observing the appropriate assay result, treated according to the present invention, include but are not limited to those

30 listed in Table 1 (for a review of such disorders, see Fishman et al., 1985, Medicine, 2d Ed., J.B. Lippincott Co., Philadelphia):



	Leukemia	
5	acute leukemia	
_	acute lymphocytic leukemia	
	acute myelocytic leukemia	
	myeloblastic	
	promyelocytic	
	myelomonocytic	
	monocytic	
	erythroleukemia	
10	chronic leukemia	
	chronic myelocytic (granulocytic)	leukemia
	chronic lymphocytic leukemia	
	Polycythemia vera	
	Lymphoma	
	Hodgkin's disease	
	non-Hodgkin's disease	
4 6		
15	Multiple myeloma Waldenström's macroglobulinemia	
	Heavy chain disease Solid tumors	
	sarcomas and carcinomas	
	fibrosarcoma	
	myxosarcoma liposarcoma	
20	chondrosarcoma	
	osteogenic sarcoma	
	chordoma	
	angiosarcoma	
	endotheliosarcoma	
	lymphangiosarcoma	
	lymphangioendotheliosarcoma	
^ E	synovioma	
25	mesothelioma	
	Ewing's tumor	
	leiomyosarcoma	
	rhabdomyosarcoma	
	colon carcinoma	
	pancreatic cancer	
	breast cancer	
30	ovarian cancer	
	prostate cancer	
	squamous cell carcinoma	
	basal cell carcinoma	
	adenocarcinoma	
	sweat gland carcinoma	
	sebaceous gland carcinoma	
35	papillary carcinoma	
3 3	papillary carcinoma papillary adenocarcinomas	
	cystadenocarcinomas	
	medullary carcinoma	
	meduliary carcinoma	



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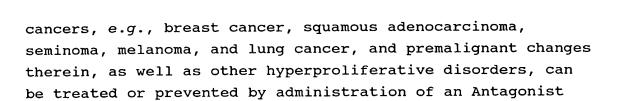
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In specific embodiments, malignancy or dysproliferative changes (such as metaplasias and dysplasias) are treated or prevented in epithelial tissues such as those in the cervix, esophagus, and lung.

Malignancies of the colon and cervix exhibit increased expression of human Notch relative to such nonmalignant tissue (see PCT Publication no. WO 94/07474 published April 14, 1994, incorporated by reference herein in its entirety). Thus, in specific embodiments, malignancies or premalignant changes of the colon or cervix are treated or prevented by administering an effective amount of an Antagonist Therapeutic, e.g., a Delta derivative, that antagonizes Notch function. The presence of increased Notch expression in colon, and cervical cancer suggests that many more cancerous and hyperproliferative conditions exhibit upregulated Notch. Thus, in specific embodiments, various

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5.8.2. NERVOUS SYSTEM DISORDERS

5 Therapeutic that antagonizes Notch function.

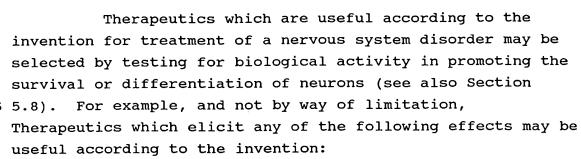
Nervous system disorders, involving cell types which can be tested as described supra for efficacy of

10 intervention with Antagonist or Agonist Therapeutics, and which can be treated upon thus observing an indication of therapeutic utility, include but are not limited to nervous system injuries, and diseases or disorders which result in either a disconnection of axons, a diminution or degeneration of neurons, or demyelination. Nervous system lesions which may be treated in a patient (including human and non-human mammalian patients) according to the invention include but are not limited to the following lesions of either the central (including spinal cord, brain) or peripheral nervous systems:

- (i) traumatic lesions, including lesions caused by physical injury or associated with surgery, for example, lesions which sever a portion of the nervous system, or compression injuries;
- (ii) ischemic lesions, in which a lack of oxygen in a portion of the nervous system results in neuronal injury or death, including cerebral infarction or ischemia, or spinal cord infarction or ischemia;
- (iii) malignant lesions, in which a portion of the nervous system is destroyed or injured by malignant tissue which is either a nervous system associated malignancy or a malignancy derived from non-nervous system tissue;
- (iv) infectious lesions, in which a portion of the nervous system is destroyed or injured as a result of infection, for example, by an

5	abscess or associated with infection by human immunodeficiency virus, herpes zoster, or herpes simplex virus or with Lyme disease, tuberculosis, syphilis; (v) degenerative lesions, in which a portion of the nervous system is destroyed or injured as a result of a degenerative process including but not limited to degeneration associated
10	with Parkinson's disease, Alzheimer's disease, Huntington's chorea, or amyotrophic lateral sclerosis; (vi) lesions associated with nutritional diseases or disorders, in which a portion of the
15	nervous system is destroyed or injured by a nutritional disorder or disorder of metabolism including but not limited to, vitamin B12 deficiency, folic acid deficiency, Wernicke
20	disease, tobacco-alcohol amblyopia, Marchiafava-Bignami disease (primary degeneration of the corpus callosum), and alcoholic cerebellar degeneration; (vii) neurological lesions associated with systemic diseases including but not limited to diabetes
25	(diabetic neuropathy, Bell's palsy), systemic lupus erythematosus, carcinoma, or sarcoidosis; (viii) lesions caused by toxic substances including
30	alcohol, lead, or particular neurotoxins; and (ix) demyelinated lesions in which a portion of the nervous system is destroyed or injured by a demyelinating disease including but not limited to multiple sclerosis, human immunodeficiency virus-associated myelopathy,
35	transverse myelopathy or various etiologies, progressive multifocal leukoencephalopathy, and central pontine myelinolysis.

15



- (i) increased survival time of neurons in culture;
- (ii) increased sprouting of neurons in culture or in vivo;
- (iii) increased production of a neuron-associated molecule in culture or in vivo, e.g., choline acetyltransferase or acetylcholinesterase with respect to motor neurons; or

Such effects may be measured by any method known in the art. In preferred, non-limiting embodiments, increased survival of neurons may be measured by the method set forth in Arakawa et al. (1990, J. Neurosci. 10:3507-3515); increased sprouting of neurons may be detected by methods set forth in Pestronk et al. (1980, Exp. Neurol. 70:65-82) or Brown et al. (1981, Ann. Rev. Neurosci. 4:17-42); increased production of neuron-associated molecules may be measured by bioassay, enzymatic assay, antibody binding, Northern blot assay, etc., depending on the molecule to be measured; and motor neuron dysfunction may be measured by assessing the physical manifestation of motor neuron disorder, e.g., weakness, motor neuron conduction velocity, or functional disability.

In a specific embodiments, motor neuron disorders that may be treated according to the invention include but are not limited to disorders such as infarction, infection, exposure to toxin, trauma, surgical damage, degenerative disease or malignancy that may affect motor neurons as well as other components of the nervous system, as well as disorders that selectively affect neurons such as amyotrophic lateral sclerosis, and including but not limited to

progressive spinal muscular atrophy, progressive bulbar palsy, primary lateral sclerosis, infantile and juvenile muscular atrophy, progressive bulbar paralysis of childhood (Fazio-Londe syndrome), poliomyelitis and the post polio syndrome, and Hereditary Motorsensory Neuropathy (Charcot-Marie-Tooth Disease).

5.8.3. TISSUE REPAIR AND REGENERATION

In another embodiment of the invention, a

10 Therapeutic of the invention is used for promotion of tissue regeneration and repair, including but not limited to treatment of benign dysproliferative disorders. Specific embodiments are directed to treatment of cirrhosis of the liver (a condition in which scarring has overtaken normal

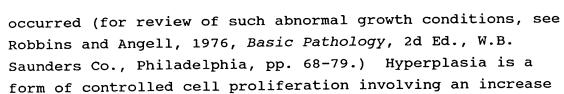
- 15 liver regeneration processes), treatment of keloid (hypertrophic scar) formation (disfiguring of the skin in which the scarring process interferes with normal renewal), psoriasis (a common skin condition characterized by excessive proliferation of the skin and delay in proper cell fate
- 20 determination), and baldness (a condition in which terminally differentiated hair follicles (a tissue rich in Notch) fail to function properly). In another embodiment, a Therapeutic of the invention is used to treat degenerative or traumatic disorders of the sensory epithelium of the inner ear.

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5.9. PROPHYLACTIC USES

5.9.1. MALIGNANCIES

The Therapeutics of the invention can be administered to prevent progression to a neoplastic or 30 malignant state, including but not limited to those disorders listed in Table 1. Such administration is indicated where the Therapeutic is shown in assays, as described supra, to have utility for treatment or prevention of such disorder. Such prophylactic use is indicated in conditions known or suspected of preceding progression to neoplasia or cancer, in particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia, or most particularly, dysplasia has



- 5 in cell number in a tissue or organ, without significant alteration in structure or function. As but one example, endometrial hyperplasia often precedes endometrial cancer. Metaplasia is a form of controlled cell growth in which one type of adult or fully differentiated cell substitutes for
- 10 another type of adult cell. Metaplasia can occur in epithelial or connective tissue cells. Atypical metaplasia involves a somewhat disorderly metaplastic epithelium. Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia; it is the most disorderly form of
- 15 non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplastic cells often have abnormally large, deeply stained nuclei, and exhibit pleomorphism. Dysplasia characteristically occurs where there exists chronic
- 20 irritation or inflammation, and is often found in the cervix, respiratory passages, oral cavity, and gall bladder.

Alternatively or in addition to the presence of abnormal cell growth characterized as hyperplasia, metaplasia, or dysplasia, the presence of one or more

- 25 characteristics of a transformed phenotype, or of a malignant phenotype, displayed in vivo or displayed in vitro by a cell sample from a patient, can indicate the desirability of prophylactic/therapeutic administration of a Therapeutic of the invention. As mentioned supra, such characteristics of a
- 30 transformed phenotype include morphology changes, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, protease release, increased sugar transport, decreased serum requirement, expression of fetal antigens, disappearance of the 250,000 dalton cell surface
- 35 protein, etc. (see also *id*., at pp. 84-90 for characteristics associated with a transformed or malignant phenotype).



In a specific embodiment, leukoplakia, a benignappearing hyperplastic or dysplastic lesion of the
epithelium, or Bowen's disease, a carcinoma in situ, are preneoplastic lesions indicative of the desirability of
prophylactic intervention.

In another embodiment, fibrocystic disease (cystic hyperplasia, mammary dysplasia, particularly adenosis (benign epithelial hyperplasia)) is indicative of the desirability of prophylactic intervention.

- or more of the following predisposing factors for malignancy is treated by administration of an effective amount of a Therapeutic: a chromosomal translocation associated with a malignancy (e.g., the Philadelphia chromosome for chronic
- 15 myelogenous leukemia, t(14;18) for follicular lymphoma, etc.), familial polyposis or Gardner's syndrome (possible forerunners of colon cancer), benign monoclonal gammopathy (a possible forerunner of multiple myeloma), and a first degree kinship with persons having a cancer or precancerous disease
- 20 showing a Mendelian (genetic) inheritance pattern (e.g., familial polyposis of the colon, Gardner's syndrome, hereditary exostosis, polyendocrine adenomatosis, medullary thyroid carcinoma with amyloid production and pheochromocytoma, Peutz-Jeghers syndrome, neurofibromatosis
- 25 of Von Recklinghausen, retinoblastoma, carotid body tumor, cutaneous melanocarcinoma, intraocular melanocarcinoma, xeroderma pigmentosum, ataxia telangiectasia, Chediak-Higashi syndrome, albinism, Fanconi's aplastic anemia, and Bloom's syndrome; see Robbins and Angell, 1976, Basic Pathology, 2d
- 30 Ed., W.B. Saunders Co., Philadelphia, pp. 112-113) etc.)

 In another specific embodiment, an Antagonist

 Therapeutic of the invention is administered to a human patient to prevent progression to breast, colon, or cervical cancer.

35



In other embodiments, a Therapeutic of the invention can be administered to prevent a nervous system disorder described in Section 5.8.2, or other disorder (e.g., 5 liver cirrhosis, psoriasis, keloids, baldness) described in Section 5.8.3.

5.10. DEMONSTRATION OF THERAPEUTIC OR PROPHYLACTIC UTILITY

The Therapeutics of the invention can be tested in vivo for the desired therapeutic or prophylactic activity. For example, such compounds can be tested in suitable animal model systems prior to testing in humans, including but not limited to rats, mice, chicken, cows, monkeys, rabbits, etc.

For in vivo testing, prior to administration to humans, any animal model system known in the art may be used.

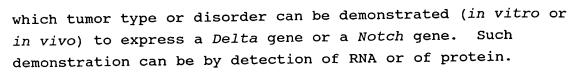
5.11. ANTISENSE REGULATION OF DELTA EXPRESSION

The present invention provides the therapeutic or prophylactic use of nucleic acids of at least six nucleotides that are antisense to a gene or cDNA encoding Delta or a portion thereof. "Antisense" as used herein refers to a nucleic acid capable of hybridizing to a portion of a Delta RNA (preferably mRNA) by virtue of some sequence

as Antagonist Therapeutics of the invention, and can be used in the treatment or prevention of disorders as described supra in Section 5.8 and its subsections.

The antisense nucleic acids of the invention can be oligonucleotides that are double-stranded or single-stranded, RNA or DNA or a modification or derivative thereof, which can be directly administered to a cell, or which can be produced intracellularly by transcription of exogenous, introduced sequences.

In a specific embodiment, the *Delta* antisense nucleic acids provided by the instant invention can be used for the treatment of tumors or other disorders, the cells of



The invention further provides pharmaceutical

5 compositions comprising an effective amount of the Delta
antisense nucleic acids of the invention in a
pharmaceutically acceptable carrier, as described infra in
Section 5.12. Methods for treatment and prevention of
disorders (such as those described in Sections 5.8 and 5.9)

10 comprising administering the pharmaceutical compositions of

The invention are also provided.

In another embodiment, the invention is directed to methods for inhibiting the expression of a *Delta* nucleic acid sequence in a prokaryotic or eukaryotic cell comprising

15 providing the cell with an effective amount of a composition comprising an antisense Delta nucleic acid of the invention.

Delta antisense nucleic acids and their uses are described in detail below.

20 5.11.1. <u>DELTA ANTISENSE NUCLEIC ACIDS</u>

The Delta antisense nucleic acids are of at least six nucleotides and are preferably oligonucleotides (ranging from 6 to about 50 oligonucleotides). In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 15

- 25 nucleotides, at least 100 nucleotides, or at least 200 nucleotides. The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar
- 30 moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci.
- 35 84:648-652; PCT Publication No. WO 88/09810, published December 15, 1988) or blood-brain barrier (see, e.g., PCT Publication No. WO 89/10134, published April 25, 1988),



hybridization-triggered cleavage agents (see, e.g., Krol et al., 1988, BioTechniques 6:958-976) or intercalating agents (see, e.g., Zon, 1988, Pharm. Res. 5:539-549).

In a preferred aspect of the invention, a *Delta*5 antisense oligonucleotide is provided, preferably of singlestranded DNA. In a most preferred aspect, such an
oligonucleotide comprises a sequence antisense to the
sequence encoding an SH3 binding domain or a Notch-binding
domain of Delta, most preferably, of human Delta. The

10 oligonucleotide may be modified at any position on its structure with substituents generally known in the art.

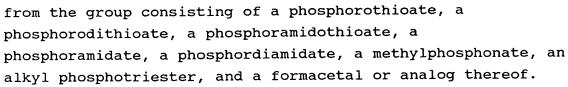
The Delta antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil,

- 20 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine,
 2-methyladenine, 2-methylguanine, 3-methylcytosine,
 5-methylcytosine, N6-adenine, 7-methylguanine,
 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil,
 beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil,
- 25 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine,
 uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil,
 queosine, 2-thiocytosine, 5-methyl-2-thiouracil,
 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil 5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v),
- 30 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

In another embodiment, the oligonucleotide comprises at least one modified sugar moiety selected from the group including but not limited to arabinose,

35 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the oligonucleotide comprises at least one modified phosphate backbone selected

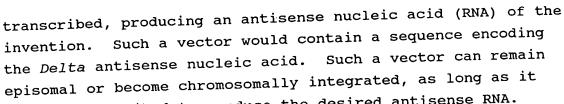


In yet another embodiment, the oligonucleotide is an α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids 10 Res. 15:6625-6641).

The oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered crosslinking agent, transport agent, hybridization-triggered cleavage agent, etc.

- oligonucleotides of the invention may be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be
- 20 synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.
- In a specific embodiment, the *Delta* antisense oligonucleotide comprises catalytic RNA, or a ribozyme (see, e.g., PCT International Publication WO 90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225). In another embodiment, the oligonucleotide is a 2'-0-
- 30 methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330).

In an alternative embodiment, the *Delta* antisense nucleic acid of the invention is produced intracellularly by 35 transcription from an exogenous sequence. For example, a vector can be introduced *in vivo* such that it is taken up by a cell, within which cell the vector or a portion thereof is

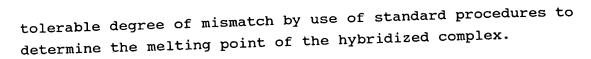


- 5 can be transcribed to produce the desired antisense RNA.

 Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence
- 10 encoding the Delta antisense RNA can be by any promoter known in the art to act in mammalian, preferably human, cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-
- of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), etc.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a *Delta* gene, preferably a human *Delta* gene. However, absolute complementarity, although preferred,

- 25 is not required. A sequence "complementary to at least a portion of an RNA," as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded Delta antisense nucleic acids, a single strand of
- 30 the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with a Delta RNA it may
- 35 contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a



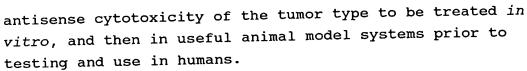
THERAPEUTIC UTILITY OF DELTA 5.11.2. ANTISENSE NUCLEIC ACIDS

The Delta antisense nucleic acids can be used to treat (or prevent) malignancies or other disorders, of a cell type which has been shown to express Delta or Notch. In specific embodiments, the malignancy is cervical, breast, or colon cancer, or squamous adenocarcinoma. Malignant, neoplastic, and pre-neoplastic cells which can be tested for such expression include but are not limited to those described supra in Sections 5.8.1 and 5.9.1. In a preferred embodiment, a single-stranded DNA antisense Delta oligonucleotide is used.

Malignant (particularly, tumor) cell types which express Delta or Notch RNA can be identified by various methods known in the art. Such methods include but are not limited to hybridization with a Delta or Notch-specific $_{20}$ nucleic acid (e.g. by Northern hybridization, dot blot hybridization, in situ hybridization), observing the ability of RNA from the cell type to be translated in vitro into Notch or Delta, immunoassay, etc. In a preferred aspect, primary tumor tissue from a patient can be assayed for Notch or Delta expression prior to treatment, e.g., by immunocytochemistry or in situ hybridization.

Pharmaceutical compositions of the invention (see Section 5.12), comprising an effective amount of a Delta antisense nucleic acid in a pharmaceutically acceptable carrier, can be administered to a patient having a malignancy which is of a type that expresses Notch or Delta RNA or protein.

The amount of Delta antisense nucleic acid which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. Where possible, it is desirable to determine the



In a specific embodiment, pharmaceutical

5 compositions comprising Delta antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, it may be useful to use such compositions to achieve sustained release of the Delta antisense nucleic acids. In a specific embodiment, it may be desirable to utilize liposomes targeted via antibodies to specific identifiable tumor antigens (Leonetti et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:2448-2451; Renneisen et al., 1990, J. Biol. Chem. 265:16337-16342).

5.12. THERAPEUTIC/PROPHYLACTIC ADMINISTRATION AND COMPOSITIONS

The invention provides methods of treatment (and prophylaxis) by administration to a subject of an effective amount of a Therapeutic of the invention. In a preferred aspect, the Therapeutic is substantially purified. The subject is preferably an animal, including but not limited to animals such as cows, pigs, chickens, etc., and is preferably a mammal, and most preferably human.

to administer a Therapeutic of the invention, e.g.,
encapsulation in liposomes, microparticles, microcapsules,
expression by recombinant cells, receptor-mediated
endocytosis (see, e.g., Wu and Wu, 1987, J. Biol. Chem.
262:4429-4432), construction of a Therapeutic nucleic acid as
part of a retroviral or other vector, etc. Methods of
introduction include but are not limited to intradermal,
intramuscular, intraperitoneal, intravenous, subcutaneous,
intranasal, epidural, and oral routes. The compounds may be
administered by any convenient route, for example by infusion
or bolus injection, by absorption through epithelial or
mucocutaneous linings (e.g., oral mucosa, rectal and
intestinal mucosa, etc.) and may be administered together



with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route,

- 5 including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an
- 10 inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be

- 15 achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous,
- 20 non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or preneoplastic tissue.
- In another embodiment, the Therapeutic can be delivered in a vesicle, in particular a liposome (see Langer, Science 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989);
- In yet another embodiment, the Therapeutic can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, supra; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507
- 35 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise



(eds.), CRC Pres., Boca Raton, Florida (1974); Controlled
Drug Bioavailability, Drug Product Design and Performance,
Smolen and Ball (eds.), Wiley, New York (1984); Ranger and
Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983);
5 see also Levy et al., Science 228:190 (1985); During et al.,
Ann. Neurol. 25:351 (1989); Howard et al., J. Neurosurg.
71:105 (1989)). In yet another embodiment, a controlled
release system can be placed in proximity of the therapeutic
target, i.e., the brain, thus requiring only a fraction of
10 the systemic dose (see, e.g., Goodson, in Medical
Applications of Controlled Release, supra, vol. 2, pp.
115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990)).

- In a specific embodiment where the Therapeutic is a nucleic acid encoding a protein Therapeutic, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that
- 20 it becomes intracellular, e.g., by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in
- 25 linkage to a homeobox-like peptide which is known to enter the nucleus (see e.g., Joliot et al., 1991, Proc. Natl. Acad. Sci. USA 88:1864-1868), etc. Alternatively, a nucleic acid Therapeutic can be introduced intracellularly and incorporated within host cell DNA for expression, by
- 30 homologous recombination.

In specific embodiments directed to treatment or prevention of particular disorders, preferably the following forms of administration are used:



Preferred Forms of Disorder <u>Administration</u> Cervical cancer Topical Gastrointestinal cancer Oral; intravenous 5 Lung cancer Inhaled; intravenous Leukemia Intravenous; extracorporeal Metastatic carcinomas Intravenous; oral Brain cancer Targeted; intravenous; intrathecal Liver cirrhosis Oral; intravenous 10 Psoriasis Topical Keloids Topical Baldness Topical Spinal cord injury Targeted; intravenous; intrathecal Parkinson's disease Targeted; intravenous; intrathecal 15 Motor neuron disease Targeted; intravenous; intrathecal Alzheimer's disease Targeted; intravenous; intrathecal

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a Therapeutic, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia 25 for use in animals, and more particularly in humans. term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, 35 particularly for injectable solutions. pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel,



sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying

- 5 agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides.
- 10 Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W.
- 15 Martin. Such compositions will contain a therapeutically effective amount of the Therapeutic, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.
- In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic
- 25 aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry
- 30 lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile
- 35 pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for



injection or saline can be provided so that the ingredients may be mixed prior to administration.

The Therapeutics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine,

10 triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the Therapeutic of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the

- 15 disorder or condition, and can be determined by standard clinical techniques. In addition, in vitro assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the
- 20 seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight.
- 25 Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from doseresponse curves derived from in vitro or animal model test systems.
- 30 Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or 35 more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental



agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

5

5.13. DIAGNOSTIC UTILITY

Delta proteins, analogues, derivatives, and subsequences thereof, Delta nucleic acids (and sequences complementary thereto), anti-Delta antibodies, have uses in 10 diagnostics. Such molecules can be used in assays, such as immunoassays, to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders affecting Delta expression, or monitor the treatment thereof. In particular, such an immunoassay is carried out by a method comprising 15 contacting a sample derived from a patient with an anti-Delta antibody under conditions such that immunospecific binding can occur, and detecting or measuring the amount of any immunospecific binding by the antibody. In a specific aspect, such binding of antibody, in tissue sections, 20 preferably in conjunction with binding of anti-Notch antibody can be used to detect aberrant Notch and/or Delta localization or aberrant levels of Notch-Delta colocalization in a disease state. In a specific embodiment, antibody to Delta can be used to assay in a patient tissue or serum 25 sample for the presence of Delta where an aberrant level of Delta is an indication of a diseased condition. Aberrant levels of Delta binding ability in an endogenous Notch protein, or aberrant levels of binding ability to Notch (or other Delta ligand) in an endogenous Delta protein may be 30 indicative of a disorder of cell fate (e.g., cancer, etc.)

body or from a subject not having the disorder.
35 The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays,

By "aberrant levels," is meant increased or decreased levels relative to that present, or a standard level representing that present, in an analogous sample from a portion of the



ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few.

Delta genes and related nucleic acid sequences and subsequences, including complementary sequences, and other toporythmic gene sequences, can also be used in hybridization

- 10 assays. Delta nucleic acid sequences, or subsequences thereof comprising about at least 8 nucleotides, can be used as hybridization probes. Hybridization assays can be used to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with aberrant changes in Delta
- 15 expression and/or activity as described supra. In particular, such a hybridization assay is carried out by a method comprising contacting a sample containing nucleic acid with a nucleic acid probe capable of hybridizing to Delta DNA or RNA, under conditions such that hybridization can occur,
 20 and detecting or measuring any resulting hybridization.

Additionally, since Delta binds to Notch, Delta or a binding portion thereof can be used to assay for the presence and/or amounts of Notch in a sample, e.g., in screening for malignancies which exhibit increased Notch expression such as colon and cervical cancers.

6. A DELTA HOMOLOG IN THE CHICK IS EXPRESSED IN PROSPECTIVE NEURONS

As described herein, we have isolated and

characterized a chick Delta homologue, C-Delta-1. We show
that C-Delta-1 is expressed in prospective neurons during
neurogenesis, as new cells are being born and their fates
decided. Our data in the chick, suggest that both the
Delta/Notch signalling mechanism and its role in neurogenesis
have been conserved in vertebrates.



We identified a chick Delta homologue, C-Delta-1, using the polymerase chain reaction (PCR) and degenerate oligonucleotide primers (Figures 1a, 1b and 2, SEQ ID NOS:1,

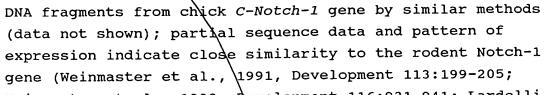
- 5 2, 3 and 4). C-Delta-1 was cloned by PCR using the degenerate oligonucleotide primers

 TTCGGITT(C/T)ACITGGCCIGGIAC (SEQ ID NO:19) and

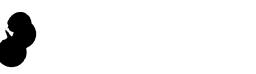
 TCIATGCAIGTICCICC(A/G)TT (SEQ ID NO:20) which correspond to the fly Delta protein sequences FGFTWPGT (SEQ ID NO:21) and
- 10 NGGTCID (SEQ ID NO:22), respectively (Vässin et al., 1987, EMBO J. 6:3431-3440; Ropczynski et al., 1988, Genes Dev. 2:1723-1735). The initial reaction used 50ng of first-strand oligo-d(T)-primed cDNA from stage 4-6 embryos, 1μg of each primer, 0.2mM dNTPs, 2U. of Taq polymerase, in 50μl of the
- 15 supplied buffer (Perkin-Elmer). 40 cycles of amplification
 were performed at 94°C/30sec; 50°C/2min; 72°C/2min.
 Amplified DNA fragments were separated on an agarose gel,
 cloned in Bluescript pKS (Stratagene) and sequenced. Two
 Delta homologs were identified, one of which (C-Delta-1) is
- 20 expressed in the nervous system. Of the homolog that is expressed in the nervous system, two variants were identified that differ at the carboxy-terminal end of the encoded protein due to an alternative splicing event at the 3' end of the C-Delta-1 gene. One encoded protein has 12 extra amino
- 25 acids at the carboxy-terminal end, relative to the other encoded protein. The sequence of the shorter encoded variant is set forth in SEQ ID NO:2. The longer variant encoded by SEQ ID NO:3 and identified by the amino acid sequence of SEQ ID NO:4, consists of the amino acid sequence of
- 30 SEQ ID NO:2 plus twelve additional amino acids at the 3' end (SIPPGSRTSLGV). The longer variant was used in the experiments described below. When tested for biological activity by injection of RNA into Xenopus oocytes, each of the variants had the same biological activity.

DNA fragments corresponding to C-Delta-1 were used to screen a stage 17 spinal cord cDNA library and several full-length clones were obtained and sequenced. We amplified

D10



- 5 Weinmaster et al., 1992, Development 116:931-941; Lardelli & Lendahl, 1993, Exp. Cell Res. 204:364-372). Sequences were analyzed using the Wisconsin GCG set of programs. The GenBank Accession number for the Chick Delta-1 mRNA is U26590. The DNA sequence of C-Delta-1 corresponds to a
- protein of 722 amino acids, structurally homologous to Drosophila Delta (Figs. 3, 4) and clearly distinct from vertebrate homologs of the Delta-related Serrate protein, which we have also cloned (data not shown). C-Delta-1 contains a putative transmembrane domain, a signal sequence
- 15 and 8 EGF-like repeats in its extracellular region (one repeat less than *Drosophila* Delta). The amino-terminal domain of C-Delta-1 is closely related to a similar domain in the fly Delta protein, described as necessary and sufficient for *in vitro* binding to Notch (Muskavitch, 1994, Dev. Biol.
- 20 166:415-430). This conserved region includes the so-called DSL motif (Fig. 4) (Henderson et al., 1994, Development 120:2913-2924; Tax et al., 1994, Nature 368:150-154), shared by all known members of the family of presumed ligands of Notch-like proteins (Delta and Serrate in Drosophila; Lag-2
- 25 and Apx-1 in Caenorhabditis elegans) (Henderson et al., 1994, Development 120:2913-2924; Tax et al., 1994, Nature 368:150-154; Fleming et al., 1990, Genes Dev. 4:2188-2201; Thomas et al., 1991, Development 111:749-761; Mello et al., 1994, Cell 77:95-106). A second cysteine-rich N-terminal
- 30 region is conserved between the fly and chick proteins, but absent from the related *C. elegans* proteins (Fig. 4). The *Xenopus Delta-1* homologue, *X-Delta-1* which encodes a protein that is 81% identical to C-Delta-1 and shows all the above structural motifs (Fig. 3), has also been cloned. The
- 35 structural conservation between the chick and fly Delta proteins, including domains identified as critical for Notch binding (Muskavitch, 1994, Dev. Biol. 166:415-430), suggests



that C-Delta-1 functions as a ligand for a chick Notch protein, and that a Delta/Notch-mediated mechanism of lateral inhibition might operate in the chick.

6.2. C-DELTA-1 AND C-NOTCH-1 EXPRESSION CORRELATES WITH ONSET OF NEUROGENESIS

During Drosophila neurogenesis, Delta is transiently expressed in neural precursors, inhibiting neighboring Notch-expressing cells from also becoming neural (Haenlin et al., 1990, Development 110:905-914; Kooh et al., 1993, Development 117:493-507). If C-Delta-1 acts similarly during chick neurogenesis, it should also be transiently expressed in neuronal precursor cells, while these are becoming determined. An analysis of C-Delta-1 expression in the developing CNS indicates that this is indeed the case.

In summary, wholemount in situ hybridization was performed. Formaldehyde fixed embryos were treated with protease and refixed with 4% formaldehyde/0.1% glutaraldehyde. Hybridization with DIG-labelled RNA probes was performed under stringent conditions (1.3xSSC, 50% formamide, 65°C, pH5) in a buffer containing 0.2% Tween-20 and 0.5% CHAPS. Washed embryos were treated with Boehringer Blocking Reagent and incubated overnight in alkaline phosphatase-coupled anti-DIG antibody. After extensive washes, embryos were stained from 30min to overnight. The embryo in Figure 5e was wax-sectioned after hybridization.

C-Delta-1 expression in the neural plate is first detected at stage 6-7 (31h, 0/1 somite), in scattered cells just anterior to the presomitic mesoderm (Fig. 5b, 5c). This region gives rise to the mid/posterior hindbrain, where the earliest differentiated CNS neurons are first detected by a neurofilament antibody at stage 9 (31h, 7-9 somites) (Sechrist & Bronner-Fraser, 1991, Neuron 7:947-963), 6h after the initial C-Delta-1 expression (Table 2).

35





TABLE 2

IADDD 2						
,		Hamburger-Hamilton Stage (nominal age in h; somite nos.)				
5	Neural tube domains	End final S-phase	Initial <i>C-Delta-1</i> expression	Initial NF expression		
10	Mid/posterior Hindbrain	4 (19h; 0)	6 (24h; 0)	9 (31h; 7-9)		
	Spinal cord, somites 5-8	6 (24h; 0)	8 (28h; 4-6)	10 (36h; 10-12)		
	Forebrain/ Midbrain	7 (25h; 1-3)	8 (28h; 4-6)	10 (36h; 10-12)		
	Spinal cord, somites 9-12	8 (28h; 4-6)	9 (31h; 7-9)	11 (43h; 13-15)		

As neurogenesis proceeds, expression of *C-Delta-1* continues to foreshadow the spatio-temporal pattern of neuronal differentiation (Table 2), spreading posteriorly along the spinal cord and anteriorly into the midbrain and forebrain (Fig 5d, 5e). For example, the most posterior expressing cells in the stage 8 spinal cord are at the level of the

cells in the stage 8 spinal cord are at the level of the prospective 6th somite, 6-8h before the first neurons at that level express neurofilament antigen (Sechrist &

shows that the appearance of *C-Delta-1* expression closely follows the withdrawal of the first neuronal precursors from the division cycle and precedes the appearance of neurofilament (NF) antigen in the resultant differentiating

- of the otic primordium; posterior hindbrain includes rhombomeres 7 and 8, and somites 1-4. Data for the timing of withdrawal from cell-division and for neurofilament expression are taken from Sechrist et al., 1991, Neuron
- 35 7:947-963. In all cases, *C-Delta-1* is expressed in scattered cells within domains of uniform *C-Notch-1* expression (Fig. 5a).





6.3. LOCALIZATION AND TIME-COURSE EXPRESSION OF C-DELTA-1

The localization and time-course of C-Delta-1 expression indicate that the gene is switched on at an early step in neurogenesis, and that the cells expressing C-Delta-1 are prospective neurons that have not yet begun to display differentiation markers. To test this hypothesis, we made use of the observations of Sechrist and Bronner-Fraser (Sechrist & Bronner-Fraser, 1991, Neuron 7:947-963) that prospective neurons are the only non-cycling cells in the They finish their final S phase 11-15h early neural tube. before expressing neurofilament antigen (Table 2) and their nuclei, after completing a last mitosis, adopt a characteristic location near the basal surface of the neuroepithelium, where all the other cell nuclei are in S-phase (Sechrist & Bronner-Fraser, 1991, Neuron 7:947-963; Martin & Langman, 1965, J. Embryol. Exp. Morphol. 14:23-35) (Fig. 6a). We labelled stage 7-9 embryos with bromodeoxyuridine (BrdU), and double-stained for BrdU incorporation and C-Delta-1 expression. 95% of the C-Delta-1-expressing cells were unlabelled, with their nuclei predominantly located near the basal surface, where most other nuclei were BrdU-labelled (Fig. 6b, 6c). 75μ l 0.1mM BrdU in PBS was dropped onto stage 7-9 embryos which were incubated at 38°C for 2-4h before fixation for in situ hybridization. 15 μ m cryostat sections were hybridized with DIG-labelled RNA probes, essentially according to the method of Strähle et al. (Strähle et al., 1994, Trends In Genet. Sci. 10:75-76). After staining, slides were washed in PBS, 30 and processed for BrdU immunodetection (Biffo et al., 1992, Histochem. Cytochem. 40:535-540). Anti-BrdU (1:1000; Sigma) was detected using FITC-coupled goat anti-mouse secondary antibody (Cappel). C-Delta-1 expression was examined by DIC microscopy, and BrdU-labelling by conventional and confocal 35 fluorescence microscopy. These results imply that C-Delta-1 is expressed in cells that have withdrawn from the cell cycle

and must indeed be prospective neurons. The few BrdU*/C-

Delta-1* cells have their nuclei outside the basal zone; these
may be cells that finished their final S-phase soon after
exposure to BrdU, moved apically to complete their final
mitosis, and switched on C-Delta-1 expression. C-Delta-1 is
also expressed in the later neural tube and peripheral
nervous system. Again, the timing of expression and the
location of the expressing cells imply that they are neuronal
precursors that have not yet begun to differentiate (data not
shown). Thus, C-Delta-1 expression appears to be the
earliest known marker for prospective neurons.

In addition, the transcription pattern of both C-Delta-1 and C-Serrate-1 overlap that of C-Notch-1 in many regions of the embryo (data not shown) which suggest that C-Notch-1, like Notch in Drosophila, is a receptor for both In particular, all three genes are expressed in 15 proteins. the neurogenic region of the developing central nervous system, and here a striking relationship is seen: the expression of both C-Serrate-1 and C-Delta-1 is confined to the domain of C-Notch-1 expression; but within this domain, 20 the regions of C-Serrate-1 and C-Delta-1 are precisely complementary. The overlapping expression patterns suggest conservation of their functional relationship with Notch and imply that development of the chick and in particular the central nervous system involves the concerted interaction of 25 C-Notch-1 with different ligands at different locations.

6.4. DISCUSSION

The Xenopus homolog of C-Delta-1 has been cloned in a similar manner. In brief, a PCR fragment of X-Delta-1 was isolated and sequenced. This fragment was then used to identify the full length clone of X-Delta-1. The X-Delta-1 expression pattern was studied. It was shown that X-Delta-1 is expressed in scattered cells in the domain of the neural plate where primary neuronal precursors are being generated, suggesting that the cells expressing X-Delta-1 are the prospective primary neurons. In addition, X-Delta-1 is also expressed at other sites and times of neurogenesis, including



the anterior neural plate and neurogenic placodes and later stages of neural tube development when secondary neurons are generated. Ectopic X-Delta-1 activity inhibited production of primary neurons; interference with endogenous X-Delta-1

- 5 activity resulted in overproduction of primary neurons.

 These results show that X-Delta-1 mediates lateral inhibition delivered by prospective neurons to adjacent cells. It was shown that ectopic expression of X-Delta-1 in Xenopus eggs suppresses primary neurogenesis, and that ectopic expression
- 10 of a truncated X-Delta-1 protein which retains only two amino acids of the cytoplasmic domain interferes with endogenous signalling and leads to extra cells developing as neuronal precursors. (Chitnis et al., Nature (in press). Preliminary evidence indicates that C-Delta-1 has a similar inhibitory
- 15 action when expressed in *Xenopus* embryos (data not shown).

 We propose that *C-Delta-1*, like its *Drosophila* and *Xenopus*counterparts, mediates lateral inhibition throughout

 neurogenesis to restrict the proportion of cells that, at any
 time, become committed to a neural fate. C-Delta-1 is
- 20 generally expressed during neurogenesis in many other sites, in both the CNS and PNS, and, for example, the developing ear. It has been shown in the CNS that C-Notch is expressed in the ventricular zone of the E5 chick hindbrain, in dividing cells adjacent to the lumen of the neural tube.
- 25 C-Delta-1 is expressed in the adjacent layer of cells, which have stopped dividing and are becoming committed as neuronal precursor cells. Thus, Delta/Notch signalling could act here, as in other neural tissues, to maintain a population of uncommitted cycling neuronal stem cells.

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7. ISOLATION AND CHARACTERIZATION OF A MOUSE DELTA HOMOLOG

A mouse *Delta* homolog, termed M-*Delta-1*, was isolated as follows:

35 Mouse Delta-1 gene

Tissue Origin: 8.5 and 9.5-day mouse embryonic RNA Isolation Method:





- random primed cDNA against above RNA a)
- PCR of above cDNA using b)

PCR primer 1: GGITTCACITGGCCIGGIACNTT (SEQ ID NO:23) [encoding GFTWPGTF (SEQ ID NO:24), a region which is specific for Delta-, not Serratelike proteins]

PCR primer 2:

GTICCICC (G/A) TT (C/T) TT (G/A) CAIGG (G/A) TT (SEQ ID NO:25) [encoding NPCKNGGT (SEQ ID NO:26), a sequence present in many of the EGF-like repeats]

Amplification conditions: 50 ng cDNA, 1 μ g each primer, 0.2 mM dNTP's, 1.8 U Taq (Perkin-Elmer) in 50 μ l of supplied buffer. 40 cycles of: 94°C/30 sec, 45°C/2 min, 72°C/1 min extended by 2 sec each cycle.

The amplified fragment was an approximately 650 base pair fragment which was partially sequenced to determine its relationship to C-Delta-1.

a mouse 11.5 day cDNA library (Clontech) was screened. Of several positive clones, one (pMDL2; insert size approximately 4 kb) included the complete protein-coding region whose DNA sequence was completely determined.

Figure 7 (SEQ ID NO:11) shows the nucleotide 'sequence of the isolated clone containing M-Delta-1 DNA.

Figure 8 (SEQ ID NO:12) shows the predicted amino acid sequence of M-Delta-1.

Figure 9 shows and amino acid alignment of the predicted amino acid sequences for M-Delta-1 and C-Delta-1.

30 Identical amino acids are boxed showing the extensive The consensus sequence is shown below sequence homology. (SEQ ID NO:13).

Expression pattern: The expression pattern was determined to be essentially the same as that observed for 35 C-Delta-1, in particular, in the presomitic mesoderm, central nervous system, peripheral nervous system, and kidney.

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A human Delta-1 homolog, termed H-Delta-1 (HDl), was isolated as follows:

human genomic library with inserts ranging in size from 100-150 kb was probed with an EcoRI fragment of the mouse Delta-1 (M-Delta-1) gene. From the library a genomic human PAC clone was isolated which hybridized to the EcoRI fragment. Next, two degenerate oligonucleotides were used to amplify by PCR a fragment of the genomic human PAC clone. The degenerate oligos were:

5' ACIATGAA(C/T)AA(C/T)CTIGCIAA(C/T)TG (SEQ ID NO:27)
[encoding TMNNLANC (SEQ ID NO:28)] and

3' AC(A/G)TAIACIGA(CYT)TG(A/G)TA(C/T)TTIGT (SEQ ID NO:29)
[encoding TKYQSVYV (SEQ ID NO:30) or

3' GC(A/G/T)ATIAC(A/G)CA(C/T)TC(A/G)TC(C/T)TT(C/T)TC

(SEQ ID NO:31) [encoding EKDECVIA (SEQ ID NO:32).

On the basis of the cDNA sequences for chicken and mouse

Delta-1, it was expected that fragments of approximately 354

and 387 base pairs would be isolated, using the 5' and the two different 3' oligos, respectively. In fact, however, two single isolates of 525 base pairs and another that was 30 base pairs smaller, as expected, were obtained. The larger isolate was sequenced by dideoxy sequencing. The nucleotide

sequence is shown in Figure 10 (SEQ ID NO:14). Also shown in Figure 10 are the predicted amino acid sequences of the amplified DNA fragment (SEQ ID NOS:15, 16, 17) for the three different readings frames. Due to sequencing errors, the full uninterrupted sequence between both primers was not identified. As a consequence, one cannot predict the amino

identified. As a consequence, one cannot predict the amino acid sequence directly from the DNA sequence obtained.

However, Figure 11 shows the amino acid sequence homology between human Delta-1 (top line) (SEQ ID NO:18) and chick Delta-1 (bottom line) as determined by eye. Because of the sequencing errors, the homology was obtained by switching

amongst the three different reading frames to identify the homologous regions.



Using the larger isolate (SEQ ID NO:14) as probe, a human fetal brain plasmid library (Clontech) was screened in an attempt to isolate full-length H-Delta-1 (HDl) genes. This yielded four positive plaques. Two of these positives 5 (HDl3 and HDl24) survived rescreening and reacted positively with a large human genomic fragment on a Southern Blot. These positive clones were subcloned by digesting with EcoRI and ligating the fragments into a Bluescript KS vector. The nucleotide sequences of the inserts were obtained by dideoxy 10 sequencing using T3 and T7 primers. The results showed that HDl24 was homologous to chicken Delta-1 at both ends; however, one end of HDl3 showed no homology. Restriction digestions with a panel of enzymes showed very similar patterns between the two clones, each of which had an insert 15 of about 2 kb, but with differences at the 3' end of HDl3.

HD13 and HD124 were cut with BstXI, XbaI, HindIII and XhoI and the restriction fragments were inserted into Bluescript KS, and then sequenced as described above to obtain internal sequence. The sequence that was obtained 20 represents the 3' about 2000 bases of HD1, extending into the 3' non-coding region. HD13 is contained within HD124; however, the added sequence at the 5' end of HD13 is likely due to a cloning artifact.

Since the sequence thus obtained did not contain

the 5' end of HDl, HDl24 was used as a probe for subsequent hybridizations in a T cell library and in another fetal brain library (Lambda Zap, Stratagene). A screen of the T cell library resulted in no positives. However, screening the Lambda-Zap library resulted in two positive clones, HDl13 and HDl18. These clones were inserted into a Bluescript KS vector using EcoRI as described above. The inserts were digested with a panel of restriction enzymes for comparison with HDl3 and HDl24, and the 5' and 3' ends were sequenced using T3 and T7 primers. HDl13 was determined to be only a small piece of cDNA that when sequenced showed no homology to any known Delta. However, HDl18 was 3 kb in length, and

included the entire sequence of \HD124 with additional 5'

sequences. A set of clones were isolated using nested deletions from HDIN8; these clones were then subjected to dideoxy sequencing using an automated sequencer. Figure 12A presents the partial nucleotide contig sequence (SEQ ID 5 NO:33) of human Delta obtained from clone HDI18. Due to sequencing errors, the full uninterrupted nucleotide sequence of human Delta was not determined. Figure 12B shows the partial nucleotide contig sequence (SEQ ID NO:33) of human Delta (top line), with the predicted amino acid sequence in three different reading frames presented below, the second line being reading frame 1 (SEQ ID NO:34), the third line being reading frame 2 (SEQ ID NO:35), and the fourth line being reading frame 3 (SEQ ID NO:36).

Sequence homology was determined by eye using the mouse Delta-1 amino acid sequence. The sequences with the greatest degree of homology to the mouse amino acid sequence are boxed in Figure 12B, and represent the predicted amino acid sequence of human Delta-1. The composite resulting amino acid sequence is shown in Figure 14. (In Figure 14, the various uninterrupted portions of the human Delta sequence are assigned respectively, SEQ ID NOS:39 through 65.) Note that due to sequencing errors, the reading frame with the greatest homology is not the same throughout the sequence and shifts at positions where there are errors in the sequence.

Further, the homology determined by eye to chicken and mouse Delta indicates that the amino acid sequence deduced from the determined human *Delta* nucleotide sequence contains all but about the N-terminal 100-150 amino acids of 30 human Delta-1.

Figure 13 presents the nucleotide sequence of mouse Delta-1 (top line, SEQ ID NO:37) and the contig nucleotide sequence of human Delta-1 as depicted in Figures 12A and 12B (second line, SEQ ID NO:33) and the nucleotide consensus sequence between mouse and human Delta (third line, SEQ ID NO:38).



Using probes containing the human Delta 5'
nucleotide sequences presented in Figure 12A, cDNA libraries
are probed to isolate the 5' end of the human Delta gene.
Primary positive clones are obtained and then confirmed as
secondary positives. The secondary positives are purified
and grown further. The DNA is then isolated and subcloned
for sequencing.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed,

- various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.
- Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

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